TITLE OF THE INVENTION
CHIMPANZEE ADENOVIRUS VACCINE CARRIERS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/538,799, filed January 23, 2004, herein incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to the field of recombinant vectors and more specifically to the production and use of recombinant replication-defective chimpanzee adenoviral vectors to elicit immune responses in mammalian hosts.

BACKGROUND OF THE INVENTION

The adenoviruses (Ads) comprise a large family of double-stranded DNA viruses found in amphibians, avians, and mammals which have a nonenveloped icosahedral capsid structure (Straus, Adenovirus infections in humans. In *The Adenoviruses.* 451-498, 1984; Hierholzer *et al., J. Infect. Dis., 158*: 804-813, 1988; Schnurr and Dondero, *Intervirology., 36*: 79-83, 1993; Jong *et al., J Clin Microbiol., 37*:3940-3945:1999). In contrast to retroviruses, adenoviruses can transduce numerous cell types of several mammalian species, including both dividing and nondividing cells, without integrating into the genome of the host cell.

Generally speaking, adenoviral DNA is typically very stable and remains episomal (e.g., extrachromosomal), unless transformation or tumorigenesis has occurred. In addition, adenoviral vectors can be propagated to high yields in well-defined production systems which are readily amenable to pharmaceutical scale production of clinical grade compositions. These characteristics and their well-characterized molecular genetics make recombinant adenoviral vectors good candidates for use as vaccine carriers. Typically, the production of recombinant adenoviral vectors relies on the use of a packaging cell line which is capable of complementing the functions of adenoviral gene products that have been either deleted or engineered to be nonfunctional.

Presently, two well-characterized human subgroup C adenovirus serotypes (i.e., hAd2 and hAd5) are widely used as the sources of the viral backbone for most of the adenoviral vectors that are used for gene therapy. Replication-defective human adenoviral vectors have also been tested as vaccine carriers for the delivery of a variety of immunogens derived from a variety of infectious agents (e.g., viruses, parasites, or bacterial pathogens) and tumor cells, including tumor-associated antigens (TAAs). Studies conducted in experimental animals (e.g., rodents, canines and nonhuman primates) indicate that recombinant replication-defective human adenoviral vectors carrying transgenes encoding immunogens

derived from the E6 and E7 oncoproteins of human papillomavirus (HPV-16) (He, Z *et al.*, (2001) *Virology*, 270:3583-3590, the rabies virus glycoprotein (Xiang, Z. *et al* (1996) Virolgy, 219:220-227), the circumsporozoite protein of *Plasmodium falciparum* Rodriguez, E. *et al.* (1997) *J. Immunol.* 158:1268-1274) as well as other heterologous antigens elicit both humoral and cell-mediated immune responses against the transgene product. Generally speaking, investigators have reported success using human adenoviral vectors as vaccine carriers in nonhuman experimental systems by either using an immunization protocols that utilizes high doses of recombinant adenoviral vectors that are predicted to elicit immune responses; or by using immunization protocols which employ the sequential administration of adenoviral vectors that are derived from different serotypes but which carry the same transgene product as boosting immunizations (Mastrangeli, *et al., Human Gene Therapy*, 7: 79-87 (1996).

However, it is predicted that vaccine carriers derived from ubiquitous human serotypes, such as types 2 and 5, will encounter preexisting humoral and cellular immunity in the human population. Thus, although replication-defective recombinant human adenoviruses have been successfully employed as vaccine carriers in experimental systems employing rodent, canine, and nonhuman primate hosts; human innate and adaptive immunity is expected to significantly limit the utility of these serotypes as vaccine carriers. This expectation is based on the fact that subgroup C, which includes type 2 and type 5, adenoviral infection is endemic in the human population. As a consequence, the majority of humans seroconvert within the first five years of life as the result of a natural infection. Thus, vectors derived from viruses that naturally infect and replicate in humans may not be optimal candidates for use as vaccine carriers.

Another problem associated with the use of human adenoviral-derived vectors is the risk that the production method used to propagate the recombinant viruses will give rise to vector stocks that are contaminated with replication competent adenovirus (RCA). This is caused by homologous recombination between overlapping sequences from the recombinant vector and the adenoviral genes that are present in the E1-complementing helper cell lines such as human 293 (Graham, F.L. *et al,* (1977) *J. Gen. Virol.* 36:59-72.) cells. The presence of RCA in vector stocks prepared for use in clinical trials constitutes a safety risk because it can promote the mobilization and spread of the replication defective virus. Spread of the defective virus can aggravate the host immune response and cause other adverse immunopathological consequences (Fallux, F. J., *et al.* Human Gene Therapy 9: 1909-1917 (1998). Accordingly, the Food and Drug Administration (FDA) and other regulatory bodies have promulgated guidelines which establish limits on the levels of RCA that can be present in vector preparations intended for clinical use. The intent of imposing RCA limits is to ensure limited exposure of patients to replicating adenovirus in compositions that are used in clinical trials.

Thus, there continues to be a need for the development of adenoviral vaccine carriers that are suitable for use in mammalian hosts which are: easy to manipulate, amenable to pharmaceutical scale

production and long term storage, capable of high-level replication in human complementation cell lines, highly immunogenic, devoid of neutralizing B cell epitopes that cross-react with the common serotypes of human adenoviruses, comply with the safety RCA standards promulgated by regulatory agencies, and which are amenable for use in prime/boost protocols that are suitable for use in humans.

SUMMARY OF THE INVENTION

The present invention relates to recombinant replication-defective adenovirus vectors derived from chimpanzee adenoviruses and methods for generating chimpanzee adenoviral vectors in human E1-expressing cell lines. The invention also provides methods for generating clinical grade vector stocks suitable for use in humans and means for using the disclosed vectors as vaccine carriers to elicit protective and/or therapeutic immune responses. The invention further provides methods for using the recombinant adenoviruses of the invention to prepare vaccine compositions designed to delivery, and direct the expression of, transgenes encoding immunogens. In one embodiment, the invention contemplates the use of the disclosed vectors as vaccine carriers for the administration of vaccines comprising transgenes encoding immunogens derived from an infectious agent. In a second embodiment, the invention contemplates the use of the disclosed vectors to prepare and administer cancer vaccines. In a particular embodiment, the invention contemplates the preparation and administration of a cancer vaccine comprising a transgene encoding a TAA.

In one aspect, the invention discloses the complete genomic sequence of five chimpanzee adenoviruses (ChAds), referred to herein as ChAd3 (SEQ ID NO: 1) (Figures 5A-5K), ChAd6 (SEQ ID NO: 2) (Figures 6A-6K, CV32 (SEQ ID NO: 3) (Figures 7A-7K), CV33 (SEQ ID NO: 4) (Figures 8A-8K), and CV23 (SEQ ID NO: 5) (Figures 9A-9J).

ChAd3 and ChAd6 represent novel adenoviruses isolated according to the methods disclosed herein. The genomes of the ChAd3 and ChAd6 are 37741 and 36648 base pairs in length, respectively. The ChAd3 hexon gene (SEQ ID NO: 41) comprises nucleotides (nt) 19086-21965 of SEQ ID NO: 1 (exclusive of stop codon) and the ChAd3 fiber gene (SEQ ID NO: 42) comprises nt 32805-34487 of SEQ ID NO: 1 (exclusive of stop codon). The ChAd6 hexon gene comprises nt 18266-21124 (SEQ ID NO: 43) of SEQ ID NO: 2 (exclusive of stop codon) and its fiber gene (SEQ ID NO: 44) comprises nt 32218-33552of SEQ ID NO:2 (exclusive of stop codon). Based on sequence homology deduced from a multiple sequence alignment of full-length hexon peptides, ChAd3 has been classified into human subgroup C and ChAd6 has been classified into human subgroup E.

The genomes of the CV32, CV33 and CV23 adenoviruses are 36,606, 36,535, and 32,020 base pairs in length, respectively. CV32 (Pan 6) (ATCC N. VR-592), CV33 (Pan 7) (ATCC N. VR-593) and CV23 (Pan 5) (Esoterix Inc.,) have all been determined to be related to human Ad4 (hAd4) (subgroup E) (Wigand, R *et al. Intervirology* 1989, 30:1-9). However, based on hexon sequence alignment CV32

has subsequently characterized as being more closely analogous to human subgroup D members than to hAd4.

In a second aspect, the invention provides nucleotide sequences for the fiber and hexon genes of 21 additional chimpanzee adenoviruses (ChAd20, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82) isolated according to the methods disclosed herein.

The fiber gene nucleotide sequences for ChAd20, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, are set forth in Figures 10-19, respectively, and are referred to herein as SEQ ID NOS: 6- 15: (SEQ ID NO: 6, ChAd20); (SEQ ID NO: 7, ChAd4); (SEQ ID NO: 8, ChAd5); (SEQ ID NO: 9, ChAd7); (SEQ ID NO: 10, ChAd9); (SEQ ID NO: 11, ChAd10); (SEQ ID NO: 12, ChAd11); (SEQ ID NO: 13, ChAd16) (SEQ ID NO: 14, ChAd17) and (SEQ ID NO: 15, ChAd19).

The fiber gene nucleotide sequences for ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 referred to herein as: (SEQ ID NO: 58, ChAd8), (SEQ ID NO: 60, ChAd22), (SEQ ID NO: 62, ChAd24), (SEQ ID NO: 64, ChAd26), (SEQ ID NO: 66, ChAd30), (SEQ ID NO: 68, ChAd31), (SEQ ID NO: 70, ChAd37), (SEQ ID NO: 72, ChAd38), (SEQ ID NO: 74, ChAd44), (SEQ ID NO: 76, ChAd63) and (SEQ ID NO: 78, ChAd82) and are set forth in the sequence listing.

The hexon gene nucleotide sequences for ChAd20, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, are set forth in Figures 21-30, respectively, and are referred to herein as SEQ ID NOS: 16-25: (SEQ ID NO: 16, ChAd20); (SEQ ID NO: 17, ChAd4); (SEQ ID NO: 18, ChAd5); (SEQ ID NO: 19, ChAd7); (SEQ ID NO: 20, ChAd9); (SEQ ID NO: 21, ChAd10); (SEQ ID NO: 22, ChAd11); (SEQ ID NO: 23, ChAd16); (SEQ ID NO: 24, ChAd17) and (SEQ ID NO: 25, ChAd19).

The hexon gene nucleotide sequences for ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 referred to herein as: (SEQ ID NO: 97, ChAd8), (SEQ ID NO: 99, ChAd22), (SEQ ID NO: 101, ChAd24), (SEQ ID NO: 103, ChAd26), (SEQ ID NO: 105, ChAd30), (SEQ ID NO: 107, ChAd31), (SEQ ID NO: 109, ChAd37), (SEQ ID NO: 111, ChAd38), (SEQ ID NO: 113, ChAd44), (SEQ ID NO: 115, ChAd63) and (SEQ ID NO: 117, ChAd82) and are set forth in the sequence listing.

In a third aspect, the invention provides amino acid sequences for the fiber and hexon proteins of 21 additional chimpanzee adenoviruses (ChAd20, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82) isolated according to the methods disclosed herein.

The fiber proteins which are disclosed and claimed here as are referred to as: (SEQ ID NO: 83, ChAd3), (SEQ ID NO: 84, ChAd6), (SEQ ID NO: 48, ChAd20), (SEQ ID NO: 49, ChAd4), (SEQ ID NO: 50, ChAd5), (SEQ ID NO: 51, ChAd7), (SEQ ID NO: 52, ChAd9), (SEQ ID NO: 53, ChAd10), (SEQ ID NO: 54, ChAd11), (SEQ ID NO: 55, ChAd16), (SEQ ID NO: 56, ChAd17), (SEQ ID NO: 57, ChAd19), (SEQ ID NO: 59, ChAd8), (SEQ ID NO: 61, ChAd22), (SEQ ID NO: 63, ChAd24), (SEQ ID NO: 65, ChAd26), (SEQ ID NO: 67, ChAd30), (SEQ ID NO: 69, ChAd31), (SEQ ID NO: 71, ChAd37), (SEQ ID NO: 73, ChAd38), (SEQ ID NO: 75, ChAd44), (SEQ ID NO: 77, ChAd63) and (SEQ ID NO: 79, ChAd82). Figures 20A-20G provides an alignment comparing the amino acid sequences of the fiber proteins disclosed and claimed herein with the amino acid sequences of the fiber proteins of: C1 (SEQ ID NO: 85), CV68 (SEQ ID NO: 86), Pan5 (alternatively referred to as CV23) (SEQ ID NO: 80), Pan6 (alternatively referred to as CV32) (SEQ ID NO: 81), and Pan7 (alternatively referred to as CV33) (SEQ ID NO: 82).

The hexon proteins which are disclosed and claimed here as are referred to as: (SEQ ID NO: 122, ChAd3), (SEQ ID NO: 123, ChAd6), (SEQ ID NO: 87, ChAd20), (SEQ ID NO: 88, ChAd4), (SEQ ID NO: 89, ChAd5), (SEQ ID NO: 90, ChAd7), (SEQ ID NO: 91, ChAd9), (SEQ ID NO: 92, ChAd10), (SEQ ID NO: 93, ChAd11), (SEQ ID NO: 94, ChAd16), (SEQ ID NO: 95, ChAd17), (SEQ ID NO: 96, ChAd19), (SEQ ID NO: 98, ChAd8), (SEQ ID NO: 100, ChAd22), (SEQ ID NO: 102, ChAd24), (SEQ ID NO: 104, ChAd26), (SEQ ID NO: 106, ChAd30), (SEQ ID NO: 108, ChAd31), (SEQ ID NO: 110, ChAd37), (SEQ ID NO: 112, ChAd38), (SEQ ID NO: 114, ChAd44), (SEQ ID NO: 116, ChAd63) and (SEQ ID NO: 118, ChAd82). Figures 31A-31J provide a comparison of the amino acid sequences of the hexon proteins disclosed and claimed herein with the amino acid sequences of the hexon proteins of: C1 (SEQ ID NO: 124), CV68 (SEQ ID NO: 125), Pan5 (alternatively referred to as CV23) (SEQ ID NO: 119), Pan6 (alternatively referred to as CV32) (SEQ ID NO: 120), and Pan7 (alternatively referred to as CV33) (SEQ ID NO: 121). A multiple sequence alignment of hexon proteins allows an artisan to perform a phylogenetic analysis of that is consistent with the proposed classification of human adenoviral serotypes (Rux, J.J., *et al* (2003) J. Virol. 77:9553-9566).

In an alternative aspect, the invention further provides 21 additional chimpanzee adenovirus isolates. Samples comprising ChAd20, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17 and ChAd19 were deposited on December 12, 2003 with the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom) as an original deposit under the Budapest Treaty. The deposits were assigned accession numbers: 03121201 (ChAd4), 03121202 (ChAd5), 03121203 (ChAd7), 03121204 (ChAd9), 03121205 (ChAd10), 03121206 (ChAd11), 03121207 (ChAd16), 03121208 (ChAd17), 03121209 (ChAd19) and 03121210 (ChAd20).

Samples comprising ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 were deposited with the ECACC (Porton

Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom) as an original deposit under the Budapest Treaty on January 12, 2005. These deposits were assigned accession numbers: 05011201 (ChAd8), 05011202 (ChAd22), 05011203 (ChAd24), 05011204 (ChAd26), 05011205 (ChAd30), 05011206 (ChAd31), 05011207 (ChAd37), 05011208 (ChAd38), 05011209 (ChAd44), 05011210 (ChAd63) and 05011211 (ChAd82).

These deposits will be maintained under the terms of the *Budapest Treaty* on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. All restrictions on the availability to the public of the deposited material will be irrevocably removed, except for the requirements specified in 37 C.F.R. §1.808(b), upon the granting of a patent.

In an additional aspect, the invention also provides replication-defective recombinant adenoviral vectors which are capable of infecting mammalian cells, preferably human cells, and directing expression of encoded transgene product(s). As demonstrated herein, the disclosed vectors are suitable for use as vaccine carriers for the delivery of transgenes comprising immunogens against which an immune response is desired. In particular embodiments, the invention provides recombinant replication-defective chimpanzee adenoviral vectors that are capable of high-level replication in human E1-expressing (i.e., packaging) cell lines. In one embodiment, the invention provides recombinant adenoviruses that are capable of replicating in PER.C6™ cells.

Generally speaking, the recombinant vectors encompassed by the invention provide vaccine carriers that will evade pre-existing immunity to the adenovirus serotypes that are typically encountered in the human population. More specifically, the recombinant vectors of the invention comprise vector backbone sequences which are shown herein to be devoid of neutralizing B epitopes that cross-react with the common serotypes of human adenoviral derived vectors.

The invention further provides group-specific shuttle vectors that include an adenoviral portion and a plasmid portion, wherein said adenoviral portion generally comprises: a) viral left end (ITR and packaging signal), part of the pIX gene and viral genome right end; and b) a gene expression cassette. The group-specific shuttle vectors are designed to exploit the nucleotide sequence homology which is observed between adenoviruses that are assigned to the same serotype subgroup (i.e., subgroups A, B, C, D or E), and can be used to manipulate the nucleotide sequences disclosed herein and/or to clone other chimpanzee adenoviruses belonging to the same subgroup generating an adenovirus pre-plasmid containing a chimp adenoviral genome deleted of E1 region.

Other aspects of this invention include host cells comprising the adenoviral vaccine vectors and/or the adenovirus pre-plasmid vectors, methods of producing the vectors comprising introducing the adenoviral vaccine vector into a host cell which expresses adenoviral E1 protein, and harvesting the

resultant adenoviral vaccine vectors. In a particular embodiment, the invention provides a method of producing a replication-defective chimpanzee adenoviral vector comprising introducing one of the disclosed adenoviral vectors into an adenoviral E-1 expressing human cell, and harvesting the resulting recombinant adenoviruses.

Another aspect of the invention also provides vaccine compositions which comprise an adenoviral vector of the invention. Compositions comprising recombinant chimpanzee adenoviral vectors may be administered alone or in combination with other viral- or non-viral-based DNA/protein vaccines. They also may be administered as part of a broader treatment regimen. These compositions can be administered to mammalian hosts, preferably human hosts, in either a prophylactic or therapeutic setting. As shown herein, administration of the disclosed vaccine compositions, either alone or in a combined modality, such as a prime boost regimen or multiple injections of serologically distinct Ad vectors results in the induction of an immune response in a mammal that is capable of specifically recognizing the immunogen encoded by the transgene.

One of the methods disclosed and claimed herein, comprises administering to a mammal (that is either naïve or primed to be immunoreactive to a target antigen), a sufficient amount of a recombinant chimpanzee adenoviral vector, containing at least a functional deletion of its wild-type E1 gene, carrying a sequence comprising a promoter capable of directing expression of a nucleotide sequence encoding the least one target antigen, wherein administration of the recombinant vector elicits (or primes) an antigen-specific immune response.

In one embodiment, the invention provides a method designed to induce an immune response (prophylactic or therapeutic) against an infectious agent (e.g., a viral or bacterial pathogen or a mammalian parasite). In a second embodiment, the invention provides a method designed to induce an immune response in a mammal that will break tolerance to a self-antigen, such as a TAA. This aspect of the invention contemplates the use of the disclosed vectors as a vaccine carrier for the preparation and administration of cancer vaccines.

Yet other embodiments and advantages of the present invention will be readily apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing which summarizes the cloning strategy used to construct a ChAd6 shuttle vector (pARS ChAd6-3).

Figure 2 is a schematic drawing which illustrates the cloning strategy used to clone the ChAd6 viral genome by homologous recombination in *E.coli* strain BJ5183.

Figure 3 is a schematic drawing illustrating the elements of various ChAd6 shuttle plasmids including: pARS ChAd6-3 GAG; pARS ChAd6-3 SEAP; pARS ChAd6-3 EGFP; and pARS ChAd6-3 NS MUT.

Figure 4 is a schematic drawing which illustrates the homologous recombination scheme utilized to clone the ChAd6 ΔE1expression vectors.

Figures 5A-5K provides the genomic nucleotide sequence of ChAd3 (SEQ ID NO: 1).

Figures 6A-6K provides the genomic nucleotide sequence of ChAd6 (SEQ ID NO: 2).

Figures 7A-7K provides the genomic nucleotides sequence of CV32 (SEQ ID NO: 3).

Figures 8A-8K provides the genomic nucleotide sequence of CV33 (SEQ ID NO: 4).

Figures 9A-9J provides the genomic nucleotide sequence of CV23 (SEQ ID NO: 5).

Figure 10 provides the nucleotide sequence of the fiber gene of ChAd20 (SEQ ID NO: 6).

Figure 11 provides the nucleotide sequence of the fiber gene of ChAd4 (SEQ ID NO: 7).

Figure 12 provides the nucleotide sequence of the fiber gene of ChAd5 (SEQ ID NO: 8).

Figure 13 provides the nucleotide sequence of the fiber gene of ChAd7 (SEQ ID NO: 9).

Figure 14 provides the nucleotide sequence of the fiber gene of ChAd9 (SEQ ID NO: 10).

Figure 15 provides the nucleotide sequence of the fiber gene of ChAd10 (SEQ ID NO: 11).

Figure 16 provides the nucleotide sequence of the fiber gene of ChAd11 (SEQ ID NO: 12).

Figure 17 provides the nucleotide sequence of the fiber gene of ChAd16 (SEQ ID NO: 13).

Figure 18 provides the nucleotide sequence of the fiber gene of ChAd17 (SEQ ID NO: 14).

Figure 19 provides the nucleotide sequence of the fiber gene of ChAd19 (SEQ ID NO: 15).

Figures 20A-20G provides a comparison of the amino acid sequences of the fiber proteins of: ChAd3, ChAd4, ChAd5, ChAd6, ChAd7, ChAd8,ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, ChAd20, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 with the reference fiber protein sequences from C1 (SEQ ID NO: 85), CV68 (SEQ ID NO: 86), PAN5 (also referred to as CV23) (SEQ ID NO: 80), PAN6 (also referred to as CV32)-(SEQ ID NO: 81) and Pan7 (also referred to as CV33) (SEQ ID NO: 82).

Figure 21 provides the nucleotide sequence of the hexon gene of ChAd20 (SEQ ID NO: 16).

Figure 22 provides the nucleotide sequence of the hexon gene of ChAd4 (SEQ ID NO: 17).

Figure 23 provides the nucleotide sequence of the hexon gene of ChAd5 (SEQ ID NO: 18).

Figure 24 provides the nucleotide sequence of the hexon gene of ChAd7 (SEQ ID NO: 19).

Figure 25 provides the nucleotide sequence of the hexon gene of ChAd9 (SEQ ID NO: 20).

Figure 26 provides the nucleotide sequence of the hexon gene of ChAd10 (SEQ ID NO: 21).

Figure 27 provides the nucleotide sequence of the hexon gene of ChAd11 (SEQ ID NO: 22).

Figure 28 provides the nucleotide sequence of the hexon gene of ChAd16 (SEQ ID NO: 23).

Figure 29 provides the nucleotide sequence of the hexon gene of ChAd17 (SEQ ID NO: 24).

Figure 30 provides the nucleotide sequence of the hexon gene of ChAd19 (SEQ ID NO: 25).

Figures 31A-31J provides a comparison of the amino acid sequences of the hexon proteins of ChAd3, ChAd4, ChAd5, ChAd6, ChAd7, ChAd8,ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, ChAd20, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 with the reference fiber protein sequences from C1 (SEQ ID NO: 124), CV68 (SEQ ID NO: 125), PAN5 (also referred to as CV23) (SEQ ID NO: 119), PAN6 (also referred to as CV32) (SEQ ID NO: 120) and Pan7 (also referred to as CV33) (SEQ ID NO: 121).

Figure 32 provides a listing of the artificial sequences SEQ ID NOS: 26-40 and SEQ ID NOS: 45 and 46, including oligomers and primers, disclosed herein.

Figure 33 is a graphic representation of the immunization break-point of ChAd vectors belonging to different serotype subgroups (i.e., subgroups C, E and D). The lowest dose eliciting a measurable immune response was determined by performing titration experiments in mice immunized with gag-expressing ChAd3, ChAd11, ChAd20, CV33, CV68, ChAd6, ChAd9, ChAd10, CV32, ChAd4, ChAd7 and ChAd16 vectors.

Figure 34 provides a graphic representation of a CEA-specific T cell response elicited in rhesus macaques immunized sequentially with a human adenoviral vector (MRKAd5 RhCEA) followed by a chimpanzee adenoviral vector (CV33 RhCEA) after 12 week interval. The immune responses were evaluated by IFN-γ ELISPOT assay, and the data illustrate the number of spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMC) following incubation in the absence (DMSO) and presence of rhesus CEA C and D peptide pools.

Figure 35 provides a phylogenetic tree of human and chimpanzee adenoviruses of deduced from a multiple sequence alignment of full-length hexon peptide sequences using PAUPSEARCH (Wisconsin Package Version 10.3, Accelrys Inc.) and visualized and manipulated with TREEVIEW.

Figure 36 is a graphic representation of immunization results obtained in response to the administration of ChAd3 and hAd5 gag vectors to mice which were pre-exposed to hAd5. Cell-mediated immunity was evaluated 3 weeks post-immunization by IFN-γ ELISPOT using purified splenocytes.

Figure 37 is a graphic representation of kinetics of anti-CEA CMI elicited in human CEA transgenic mice immunized with ChAd3hCEA and Ad5hCEA. CMI was evaluated by ICS of PBMC stimulated with CEA peptide pool. The results are expressed as % of $IFN\gamma^+$ $CD8^+$/total PBMC.

Figures 38 A-D is a graphic representation of the efficiency of infection of different human primary cells exposed to moi 50, 250 and 1250 of different ChAd vectors expressing EGFP and belonging to different subgroups (B, C, D, E). The results are expressed as % of fluorescent cells /on total cells.

DETAILED DESCRIPTION OF THE INVENTION

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "cassette" refers to a nucleic acid molecule which comprises at least one nucleic acid sequence that is to be expressed, along with its transcription and translational control sequences. Changing the cassette, will cause the vector into which is incorporated to direct the expression of different sequence or combination of sequences. In the context of the present invention, the nucleic acid sequences present in the cassette will usually encode an immunogen. Because of the restriction sites engineered to be present at the 5' and 3' ends, the cassette can be easily inserted, removed or replaced with another cassette.

The term "*cis*-acting element" refers to nucleotide sequences which regulate genes to which they are attached. *Cis*-acting elements present in DNA regulate transcription, and those transcribed into mRNA can regulate RNA processing, turnover and protein synthesis.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The term "promoter" refers to a recognition site on a DNA strand to which an RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences such as enhancers, or inhibiting sequences such as silencers.

The term "pharmaceutically effective amount" refers to an amount of recombinant adenovirus that is effective in a particular route of administration to transduce host cells and provide sufficient levels of transgene expression to elicit an immune response.

The term "replication-competent" recombinant adenovirus (AdV) refers to an adenovirus with intact or functional essential early genes (i.e., E1A, E1B, E2A, E2B and E4). Wild type adenoviruses are replication competent.

The term "replication-defective" recombinant AdV refers to an adenovirus that has been rendered to be incapable of replication because it has been engineered to have at least a functional deletion, or a complete removal of, a gene product that is essential for viral replication. The recombinant chimpanzee adenoviral vectors of the invention are replication-defective.

The term "mammalian" refers to any mammal, including a human being.

The term "percent sequence identity" or "identical" in the context of nucleic acid sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the genome (e.g., about 36 kbp), the full-length of an open reading frame of a gene, protein, subunit, or enzyme [see, e.g., the tables providing the adenoviral coding regions], or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, e.g. of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. Similarly, "percent sequence identity" may be readily determined for amino acid sequences, over the full-length of a protein, or a fragment thereof. Suitably, a fragment is at least about 8 amino acids in length, and may be up to about 700 amino acids. Examples of suitable fragments are described herein.

Identity is readily determined using such algorithms and computer programs as are defined herein at default settings. Preferably, such identity is over the full length of the protein, enzyme, subunit, or over a fragment of at least about 8 amino acids in length. However, identity may be based upon shorter regions, where suited to the use to which the identical gene product is being put.

In general, adenoviral constructs, gene constructs are named by reference to the genes contained therein. For example, "pChAd3 ΔE1gag" refers to a plasmid construct which comprises a ChAd3 chimpanzee adenoviral genome deleted of the E1 region. In this plasmid, the E1 region is replaced by an immunogen expression cassette comprising an HIV gag gene under the control of a human CMV promoter followed by a bovine growth hormone polyadenylation signal. Similarly, pCV33DE1-E3 NSmut, refers to a second plasmid construct disclosed herein which comprises a CV33 chimpanzee adenoviral genome, deleted of the E1 and E3 regions, which is replaced by an immunogen expression cassette comprising HCV non-structural genes under the control a human CMV promoter followed by a bovine growth hormone polyadenylation signal.

The abbreviation "Ag" refers to an antigen.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

Adenoviruses (Ads) are noneveloped, icosahedral viruses that have been identified in several avian and mammalian hosts. Human Ads (hAd) belong to the Mastadenovirus genus which includes all known human and many Ads of animal (e.g., bovine, porcine, canine, murine, equine, simian

and ovine) origin. Human adenoviruses are divided into six subgroups (A-F) based on a number of biological, chemical, immunological and structural criteria which include hemagglutination properties of rat and rhesus monkey erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity (Straus, 1984, In *The Adenoviruses*, ed. H. Ginsberg, pps. 451-498, New York: Plenus Press, and Horwitz, 1990 In *Virology*, eds. B.N. Fields and D.M. Knipe, pps. 1679-1721). To date, 51 distinct serotypes have been recognized and grouped into subgroups on the basis of their hemagglutination properties and biophysical and biochemical criteria.

The adenoviral virion has an icosahedral symmetry and, depending on the serotype, a diameter of 60-90 nm. The icosahedral capsid consists three major proteins, hexon (II), penton base (III) and a knobbed fiber (IV) as well as a number of minor proteins (i.e., VI, VIII, IX, IIIa and IVa2) (W.C. Russel, *J. Gen. Virol.*, 81: 2573-2604 (2000). One aspect of the preexisting immunity that is observed in humans is humoral immunity, which can result in the production and persistence of antibodies that are specific for viral proteins. The humoral response elicited by adenovirus is mainly directed against the major structural proteins: hexon, penton and fiber.

Published reports have established that titers comprising antibodies against multiple serotypes are common (Dambrosio, E. (1982) *J. Hyg.* (London) 89: 209-219) and that a substantial portion of the preexisting titers have neutralizing activity. Neutralizing immunity to adenovirus is type specific, and infection with a particular serotype of adenovirus confers immunity only to that serotype. Several reports have suggested that antibodies directed towards the hexon are the strongest and the most neutralizing (Toogood, C.I.A., Crompton, J. and Hay R.T. (1992) *J.Gen. Virol.* 73, 1429-1435). Therefore, it is reasonable to assume that the epitopes responsible for type-specific neutralization are located within seven hypervariable regions identified by alignment of the hexon sequences deriving from different serotypes. (Crawford-Miksza, L and D.P.Schnurr. (1996) *J.Virol.*70:1836-1844).

A direct correlation between the presence of type-specific neutralizing antibodies and the inability to elicit an immune response with a vector based on the same serotype has been established by different methods including the passive transfer of immune sera from treated to naïve animals. Generally speaking, preexisting humoral immunity for a specific viral serotype reduces the therapeutic efficacy of the vector administration. Moreover, the administration of a vector based on a specific viral serotype elicits an immune-response against the vector that prevents the re-administration of the same serotype.

In a particular embodiment, the invention provides a method of circumventing the adverse effects associated with the consequences of preexisting immunity to common serotypes of hAds. More specifically, the invention contemplates the use of chimpanzee adenoviral vectors characterized by a serotype that does not circulate in humans. Accordingly, the invention provides adenoviral (Chad) vectors which lack neutralizing B-cell epitopes that cross react with those of common human serotypes as a vaccine carrier.

Although it has been reported that adenoviral-specific cell mediated immunity (CMI) can be cross-reactive, vaccination studies based on repeated injections of multiple serotypes demonstrated a higher efficiency than immunization schedules based on a single vector. These experiments further demonstrate that the main limitation of a vector administration for vaccine purposes is the humoral pre-existing immunity against the vector. Potential solutions to the problems associated with the use of a human adenovirus as a vaccine carrier include the administration of a higher dose of an adenovirus (e.g., a subgroup C serotype) that is predicted to encounter a preexisting immune response, and the use of vectors based on rare human serotypes. However, the use of higher doses of vaccine increases the cost of the vaccine and risk of undesirable side effects and the results of preclinical testing suggest that human alternate serotypes are less immunogenic than hAd5 and hAd6.

In an attempt to avoid the problems of host humoral and cellular immune responses against the adenoviral backbone elements of the vector, and to minimize the risk of using human adenovirus-derived vector stocks that may be contaminated with replication-competent adenoviruses (RCA), several nonhuman adenoviruses have been characterized and developed as vaccine carriers (Soudois, C. *et al* (2000) *J. Virology*, 74:10639-10649; Farina, S.F. *et al* (2001) *J. Virology*, 75:11603-11613; Cohen, C.J. *et al* (2002) *J. Gen. Virology*, 83:151-155.) The premise underlying the use of nonhuman adenoviral sequences to circumvent the problems associated with preexisting immunity is based on the observation that neutralizing antibodies to common human adeonvirus serotypes are unlikely to cross-neutralize nonhuman viruses. However, the incompatibility of viral and cellular factors imposes a practical limitation on the vast majority of alternative vector systems (bovine, ovine, canine) which are characterized by the disadvantage of having to be propagated in non-human cell lines.

Wilson *et al.* have published a report describing the characterization of a replication-defective vector based on chimpanzee adenovirus type 68 (CV68) C68, which was originally isolated from a mesenteric lymph node of a chimpanzee (Basnight, M., *et. al.* (1971) *Am. J. Epidemiol.* 94:166-171.), CV68 was fully sequenced and found to be similar in overall structure to human adenoviruses (Farina, S. F. *et al., J. Virol.* 75(23): 11603-11613 (2001). The genome of the virus is 36,521 base pairs in length and has been described as being most similar to subgroup E of human adenoviruses, with 90% identity to most human Ad4 open reading frames that have been sequenced. The CV68 ITRs are 130 base pairs in length, and all of the major adenoviral early and late genes are present. CV68 is characterized by a serotype that does not circulate in humans and which lacks neutralizing B cell epitopes that cross-react with those of common human serotypes. Although Chimpanzee adenonviruses are similar to human adenoviruses cross-reactive neutralizing immunity against chimpanzee serotypes has not been documented in humans (Farina, S. F. *et al. J. Virol.* (2001) 75(23):11603-13).

The recombinant vectors derived from CV68 are described as being sufficiently similar to human serotypes to support transduction of cells expressing the coxsackievirus and adenovirus receptor

(Cohen, C. *et al., J. Gen. Virol.* 83: 151-155 (2002). Significantly, CV68 is characterized by a sufficient level of similarity to human adenoviruses to support its replication 293 cells which harbor E1 from human adenovirus type 5 (Farina, S. F. *et al., J. Virol.* 75(23): 11603-11613 (2001). Furthermore, based on the observation that the flanking sequences of the human serotype 5 E1 are nonhomologous with those of the CV68-derived vector sequences, it is predicted that homologous recombination will not occur. Thus, it has been predicted that there is a low likelihood that CV68-derived vaccine stocks will be contaminated with RCA.

The same group of investigators subsequently reported the use of CV68-derived adenoviral sequences as a vaccine carrier for induction of antibodies to the rabies virus glycoprotein in mice. A replication-defective version of CV68 was created by replacing the E1A and E1B genes with a minigene cassette. Mice immunized with an E1-deletion-containing adenoviral recombinant (AdC68rab.gp) comprising a transgene product encoding the rabies virus glycoprotein developed protective immunity to rabies virus and remained resistant to challenge with an otherwise lethal dose of rabies virus (Xiang, Z *et al., J. Virol.* 76(5): 2667-2675 (2002). A second CV68 construct expressing a codon-optimized, truncated form of gag of HIV-1 was recently reported to induce a vigorous gag-specific $CD8^+$ T cell response in mice. The vaccine-induced response was shown to provide protection to challenge with a vaccinia gag recombinant virus (Fitzgerald, J. C. *et al., J. Immunol.* 170: 1416-1422 (2003). Experimental vaccination of mice preimmunized to human adenovirus serotype 5 with CV68gag or Ad5gag vectors demonstrated a more pronounced reduction of gag-specific T cells and protection against viral challenge elicited by Ad5 than by CV68 vaccine. The reduction in efficacy of C68gag vaccine was attributed to a cross-reactivity of Ad5-specific CD8+ T cells (*Id.*).

Considered together this data suggests that simian-derived replication-defective adenoviral vectors may be more suitable for use as human vaccine carriers than vectors based on common human serotypes. As shown herein, the results of experiments in which mice that were strongly immunized against human Ad5 (Figure 36) can be immunized with ChAd3-gag adenoviral vectors indicate the preexisiting anti-human Ad5 immunity did not reduce the gag-specific CMI response elicited by the ChAd vectors. These results are consistent with the conclusion that human Ad5 cross-reactive B and T-cell epitopes are not present in ChAd3- or ChAd6 vectors.

Generally speaking, the adenoviral genome is very well characterized and despite the existence of several distinct serotypes, there is some general conservation in the overall organization of the adenoviral genome with specific functions being similarly positioned. The nucleotide sequences of the chimpanzee adenoviruses C1 and CV68 disclosed by Wilson *et al.*, and the location of the E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4 and L5 genes of each virus are provided in U.S. Patent No. 6,083,716 (Chimpanzee Adenovirus Vectors), and PCT published application WO 03/000851 (Methods for Rapid

Screening of Bacterial Transformants and Novel Simion Adenoviral Proteins), the teachings of which are incorporated herein by reference.

Each extremity of the adenoviral genome comprises a sequence known as an inverted terminal repeat (ITRs), which is necessary for viral replication. The virus also comprises a virus-encoded protease, which is necessary for processing some of the structural proteins required to produce infectious virions. The structure of the adenoviral genome is described on the basis of the order in which the viral genes are expressed following host cell transduction. More specifically, the viral genes are referred to as early (E) or late (L) genes according to whether transcription occurs prior to or after onset of DNA replication. In the early phase of transduction, the E1, E2, E3 and E4 genes of adenovirus are expressed to prepare the host cell for viral replication. The virus can be rendered replication defective by deletion of the essential early-region 1(El) of the viral genome. Brody *et al*, 1994 *Ann N Y Acad Sci.*, 716:90-101. During the late phase, expression of the late genes L1-L5, which encode the structural components of the virus particles is switched on. All of the late genes are under the control of a single promoter and encode proteins including the penton (L2), the hexon (L3), the 100 kDa scaffolding protein (L4), and the fiber protein (L5), which form the new virus particle into which the adenoviral DNA becomes encapsidated. Depending on the serotype of the virus, 10,000-100,000 progeny adenovirus particles can be generated in a single host cell. Ultimately, the adenoviral replication process causes lysis of the cells.

The replication-defective adenoviral vectors disclosed herein were constructed by deletion of specific nucleotide sequences from the disclosed chimpanzee nucleic acid sequences and insertion of sequences derived other DNA sequences that are useful for transgene insertion, expression or other genetic manipulations. Accordingly, the recombinant chimpanzee adenoviruses described herein may contain adenoviral sequences derived from one or more chimpanzee adenoviruses, or sequences from a chimpanzee adenovirus and from a human adenovirus. Suitable polynucleotide sequences can be produced recombinantly, synthetically or isolated from natural sources. Adenoviral sequences suitable for use in particular aspects of the invention include sequences which lack neutralizing B-cell epitopes that are cross-reactive with common human serotypes.

At a minimum, the recombinant chimpanzee adenovirus (e.g., vector) of the invention contain the chimpanzee adenovirus *cis*-acting elements necessary for replication and virion encapsidation, in combination with at least one immunogen expression cassette. Typically, the *cis*-acting elements flank the expression cassette which comprises a transgene that encodes at least one antigen. More specifically, the vectors of the invention contain the requisite *cis*-acting 5' inverted terminal repeat (ITR) sequences of the adenoviruses (which function as origins of replication), 3' ITR sequences, packaging/enhancer domains, and a nucleotide sequence encoding a heterologous molecule. Regardless of whether the recombinant vector comprises only the minimal adenoviral sequences or an entire adenoviral genome

with only functional deletions in particular genes (e.g., the E1 and/or E3 or E4 regions), the vectors of the invention comprise a chimpanzee adenovirus capsid.

Generally, speaking the adenoviral vectors disclosed herein comprise a replication-defective adenoviral genome, wherein the adenoviral genome does not have a functional E1 gene, and an immunogen expression cassette which comprises: a) a nucleic acid encoding at least one immunogen against which an immune response is desired; and b) a heterologous (i.e., with respect to the adenoviral sequence) promoter operatively linked to the nucleic acid sequence encoding the immunogen(s); and a transcription terminator.

More specifically, the invention provides replication-defective vectors that consist of a recombinant adenoviral genome that is devoid of at least one early gene selected from the group consisting of E1, E2, E3, and E4. In one embodiment, a replication-defective vector is prepared by replacing, or disrupting, the E1 gene of one of the adenoviral isolates disclosed herein (e.g., ChAd3, ChAd6, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19,ChAd20, ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 or ChAd82) with an immunogen expression cassette. For example, a vector can be prepared by deleting/disrupting the E1 gene of ChAd 3 (SEQ ID NO:1) or ChA6 (SEQ ID NOS: 2). Alternatively, a replication-defective vector can be prepared from any one of the other adenovirus isolates disclosed herein, including ChAd3, ChAd6, ChAd4, ChAd5, ChAd7, ChAd9, ChAd10, ChAd11, Chad16, Chad17, ChAd19, ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 or ChAd20. In other embodiments, replication-defective vectors of the invention comprises an adenoviral genome derived from one of the ChAds disclosed herein that has been optionally engineered to lack a functional E3 gene. It is to be understood that the chimpanzee adenoviral sequences disclosed herein can be rendered replication-defective by either completely removing an early gene or by rendering the gene inoperative or nonfunctional.

It is to be understood that the invention encompasses vectors that are characterized as having modifications, such as a "functional deletion" which destroys the ability of the adenovirus to express one or more selected gene products. The phrase "functional deletion" as used herein broadly encompasses modifications that have the effect of rendering a particular gene product nonfunctional. Generally speaking, functional deletions take the form of a partial or total deletion of an adenoviral gene. However, one of skill in the art will readily acknowledge that other manipulations, including but not limited to making a modification which introduces a frame shift mutation, will also achieve a functional deletion. For example, the recombinant chimpanzee adenoviral vectors of the invention can be rendered replication-defective by introducing a modification that is designed to interfere with, or to functionally delete, the ability of the virus to express adenoviral E1A and/or E1B.

It is well-known that replication-defective adenoviral vectors can be obtained by introducing a modification that is designed to interfere with, or to functionally delete the expression of one o more genes from the group of E2 genes. More in detail, a replication-defective vector can be constructed by inactivating the polymerase gene, or the pre-terminal protein gene or the DNA binding protein gene. Moreover deletion or inactivation of genes expressed by E4 region is an alternative strategy to construct replication-defective chimp Ad vectors. Early gene deletion or inactivation can be combined in order to produce more attenuated vectors. Alternatively, replication-defective ChAd vectors can also comprise additional modifications in other viral genes, such as the late genes L1 through L5. In addition, novel adenoviral vaccine carriers can be generated by combining hexon and fiber genes obtained from different serotypes. The utilization of a hexon and fiber gene shuffling strategy will also allow an investigator to change the biological properties of a ChAd and facilitate the production of vectors with a different tropism or with new serological characteristics.

It is to be understood that the present invention encompasses recombinant adenoviral vectors comprising deletions of entire genes or portions thereof which effectively destroy the biological activity of the modified gene either alone or in any combination. For example, recombinant simian adenoviruses can be constructed which have a functional deletion of the genes expressed by E4 region, although as shown herein it may be desirable to introduce the heterologous Ad5 E4 sequence into the vector in combination with the functional deletion of an E1 gene. Alternatively, the function of the adenoviral delayed early E3 gene may be eliminated; however because the function of E3 is not necessary for the production of a recombinant adenoviral particle it is not necessary to replace this gene product in order to produce a recombinant that is capable of packaging a virus useful in the invention.

In one embodiment of this invention, the replication- defective adenoviral vector used is a chimpanzee subgroup C adenovirus containing deletions in E1 and optionally in E3. For example, for ChAd3, a suitable E1 deletion/disruption can be introduced in the region from bp 460 to bp 3542 (with reference to SEQ ID NO: 1). For ChAd6, a suitable E1 deletion/disruption can be introduced in the region from bp 457 to bp 3425 (with reference to SEQ ID NO: 2). For CV32, the E1 deletion is preferably from bp 456 to bp 3416 (with reference to SEQ ID NO: 3); for CV33, the E1 deletion is preferably from bp 456 to bp 3425 (with reference to SEQ ID NO: 4) and for CV23, the E1 deletion is preferably from bp 456 to bp 3415 (with reference to SEQ ID NO: 5). E3 deletions for CV32 and CV33 are preferably from bp 27446 to bp 31911 (with reference to SEQ ID NO: 3); from bp 27146 to bp 31609 (with reference to SEQ ID NO: 4) respectively. Those of skill in the art can easily determine the equivalent sequences for other chimpanzee isolates based on sequence homologies and multiple sequence alignments.

One of skill in the art will readily acknowledge that in order to construct an E1-deleted adenoviral vector a number of decisions must be made regarding the structure of the vector backbone and

the composition of the nucleic acid sequence comprising the transgene. For example, an investigator must determine if the size of the E1 deletion will accommodate the size of the transgene. If not, then additional deletions will have to be introduced into the backbone of the vector.

The nucleic acid sequence embodying the transgene can be a gene, or a functional part of a gene and will typically exist in the form of an expression cassette. Typically a gene expression cassette includes: (a) nucleic acid encoding a protein or antigen of interest; (b) a heterologous promoter operatively linked to the nucleic acid encoding the protein; and (c) a transcription termination signal. The nucleic acid can be DNA and/or RNA, can be double or single stranded. The nucleic acid can be codon-optimized for expression in the desired host (*e.g.*, a mammalian host).

Decisions must also be made regarding the site within the backbone where the transgene will be introduced and the orientation of the transgene. More specifically, the transgene can be inserted in an E1 parallel (transcribed 5' to 3') or anti-parallel (transcribed in a 3' to 5' direction relative to the vector backbone) orientation. In addition, appropriate transcriptional regulatory elements that are capable of directing expression of the transgene in the mammalian host cells that the vector is being prepared for use as a vaccine carrier in need to be identified and operatively linked to the transgene. "Operatively linked" sequences include both expression control sequences that are contiguous with the nucleic acid sequences that they regulate and regulatory sequences that act in *trans*, or at a distance to control the regulated nucleic acid sequence.

Regulatory sequences include: appropriate expression control sequences, such as transcription initiation, termination, enhancer and promoter sequences; efficient RNA processing signals, such as splicing and polyadenylation signals; sequences that enhance translation efficiency (e.g., Kozak consensus sequences); sequences that enhance protein stability, and optionally sequences that promote protein secretion. Selection of these and other common vector elements are conventional and many suitable sequences are well known to those of skill in the art (see, e.g., Sambrook *et al*, and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27 and Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989).

In specific embodiments, the promoter is a heterologous promoter (i.e., with respect to the adenovirus sequences) which is recognized by an eukaryotic RNA polymerase. In a preferred embodiment, the promoter is a "strong" or "efficient" promoter. An example of a strong promoter is the immediate early human cytomegalovirus promoter (Chapman *et al*, 1991 *Nucl. Acids Res* 19:3979-3986, which is incorporated by reference). The human CMV promoter can be used without (CMV) or with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a number of other known promoters, such as the strong immunoglobulin, or other eukaryotic gene promoters may be used, including the EF1 alpha promoter, the murine CMV promoter, Rous sarcoma virus (RSV) promoter, SV40 early/late promoters and the beta-actin promoter.

Further examples of promoters that can be used in the present invention are the strong immunoglobulin promoter, the EF1 alpha promoter, the murine CMV promoter, the Rous Sarcoma Virus promoter, the SV40 early/late promoters and the beta actin promoter, albeit those of skill in the art can appreciate that any promoter capable of effecting expression in the intended host can be used in accordance with the methods of the present invention. The promoter may comprise a regulatable sequence such as the Tet operator sequence. Sequences such as these that offer the potential for regulation of transcription and expression are useful in instances where repression of gene transcription is sought.

Suitable gene expression cassettes will also comprise a transcription termination sequence. A preferred transcriptional terminator is the bovine growth hormone terminator. The promoter/transcription termination combination of CMVintA-BGH terminator is particularly preferred although other promoter/terminator combinations may also be used. As shown herein, the bovine growth hormone termination/polyadenylation signal (bGHpA) or short synthetic polyA signal (SPA) of 50 nucleotides in length defined as follows:

AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTTGTGTG (SEQ ID NO: 26).

Generally speaking, exemplify suitable termination sequences. The polyA signal is inserted following the nucleic acid sequence which comprises the transgene and before the 3' adenovirus ITR sequence.

The recombinant adenoviral vectors described herein may contain adenoviral sequences derived from one or more strain of adenovirus. Suitable sequences may be obtained from natural sources, produced recombinantly, synthetically, or by other genetic engineering or chemical methods. In a particular embodiment, the recombinant chimpanzee adenovirus is a chimeric recombinant comprising non-chimpanzee adenoviral polynucleotide sequences. Suitable non-chimpanzee adenoviral sequences can be obtained from human adenoviral strains. For example, the native E4 region can be replaced by hAd5 E4 (Ad5 nt 32816 to nt 35619) or by Ad5E4orf6 (Ad5 nt 33193 to nt 34077) (Ad5 GenBank Accession No: M73260).

Generally speaking, the immunogen (antigenic molecule) delivered by the recombinant adenoviral vector of the invention comprises a polypeptide, protein, or enzyme product which is encoded by a transgene in combination with a nucleotide sequence which provides the necessary regulatory sequences to direct transcription and/or translation of the encoded product in a host cell. The composition of the transgene depends upon the intended use of the vector. For example, if the immunogenic composition is being designed to elicit an antibody response or a cell-mediated immune response in a mammalian host which is specific for an infectious agent, then it is appropriate to utilize a nucleic acid sequence encoding at least one immunogenic product that is predicted to confer pathogen-specific immunity to the recipient. Alternatively, if the composition is being prepared for use as a cancer vaccine, a suitable transgene may comprise an immunogenic portion of a self-antigen, such as a TAA, which has

been selected with the goal of eliciting a protective immune response of sufficient potency to both break host tolerance to a particular TAA and to elicit a long-lived (e.g., memory) response that will be sufficient to prevent the initiation of cancer or to prevent tumor progression. Accordingly, suitable immunogenic gene products may be obtained from a wide variety of pathogenic agents (such as, but not limited to viruses, parasites, bacteria and fungi) that infect mammalian hosts, or from a cancer or tumor cell. Although, the invention is illustrated herein with a particular set of test immunogens it is to be understood that the invention is not limited to the use of the antigens exemplified herein. More specifically, the invention contemplates the use of both heterologous and self-antigens as immunogens, including but not limited to TAAs.

In one embodiment, the invention provides an immunogenic composition (e.g., a vaccine) for inducing an immune response against antigens (i.e., immunogens) expressed by an infectious agent. For example, it is desirable to elicit an immune response against a virus infecting humans and/or non-human animal species. Examples of virus families against which a prophylactic and/or therapeutic immune response would be desirable include the *Picornaviridae* family which includes six different genera such as Aphtovirus, Cardiovirus, Enterovirus, Hepatovirus, Parechovirus, Rhinovirus. Examples of Picornavirus against which an immune response would be desirable are: Foot-and-mouth disease viruses, Encephalomyocarditis viruses, Polioviruses, Coxackieviruses, Human hepatitis A virus, Human parechoviruses, Rhinoviruses. *Caliciviridae* family includes different genera associated with epidemic gastroenteritis in humans caused by the Norwalk group of viruses and other syndromes in animals like the hemorrhagic disease in rabbits associated with rabbit hemorrhagic disease virus or respiratory disease in cats caused by feline calicivirus.

Another family of viruses, against which it may be desirable to elicit an immune response is the *Astroviridae* which comprises viruses isolated from humans as well as many different animal species. Human astroviruses are associated with gastroenteritis and young children diarrhea. Alternatively, it may be desirable to confer mammalian hosts with immunity to members of the *Togaviridae* family of viruses which comprises two genera: alphavirus and rubivirus. Alphaviruses are associated with human and veterinary diseases such as arthritis (i.e. Chikungunya virus, Sindbis virus) or encephalitis (i.e. Eastern Equine Encephalitis Virus, Western Equine Encephalitis Virus).

Rubella virus provides an alternative viral target against which is the only member of the Rubivirus genus is responsible for outbreaks of a mild exanthematic disease associated with fever and lymphoadenopathy. Rubella virus infection is also associated with fetus abnormalities when acquired by mother during in early pregnancy. *Flaviviridae* is another virus family consisting of three genera: the flaviviruses, the pestiviruses and the hepaciviruses that includes important human as well as animal pathogens. Many of the flavivirus genus members are arthropod-borne human pathogens causing a variety of diseases including fever, encephalitis and hemorrhagic fevers. Dengue Fever Viruses, Yellow

Fever Virus, Japanese Encephalitis Virus, West Nile Fever Virus, Tick-borne Encephalitis Virus are pathogens of major global concern or of regional (endemic) concern. Pestivirus genus includes animal pathogens of major economic importance such as Bovine Viral Diarrhea Virus, Classical Swine Fever Virus, Border Disease Virus. Hepatitis C Virus is the only member of the Hepacivirus genus responsible for acute and chronic hepatitis. HCV proteins expressed by a recombinant adenovirus can elicit a protective as well as therapeutic immune response limiting the consequences of a viral infection affecting 170 million people worldwide.

Alternatively, antigens derived from members of the *Coronaviridae* family can be expressed by recombinant adenovirus vectors in order to obtain protection against infection. Protection against the severe acute respiratory syndrome coronavirus (SARS-Co Virus) can be obtained by immunizing with one or more chimpanzee adenovirus chosen from the group including ChAd3, 4, 5,6, 7,9,10,11,16,17,19, 20, ChAd8, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82 expressing one or more SARS-CoV protein including without limitations nucleocapsid (N) protein, polymerase (P) protein, membrane (M) glycoprotein, spike (S) glycoprotein, small envelope (E) protein or any other polypeptide expressed by the virus. *Rhabdoviridae* family members including rabies virus can be target of recombinant vaccine expressing viral proteins.

Other possible targets include the *Filoviridae* family comprising Ebola-like viruses and Marburg-like viruses genera, that is responsible of outbreaks of severe hemorrhagic fever; the *Paramyxoviridae* family comprising some of the most prevalent virus known in humans like measles, respiratory syncytial, parainfluenza viruses and viruses of veterinary interest like Newcastle disease and rinderpest viruses; the *Orthomyxoviridae* family including Influenza A,B,C viruses; *Bunyaviridae* family mainly transmitted by arthropod to vertebrate hosts comprising important human pathogens like Rift valley fever, Sin Nombre, Hantaan, Puumala viruses; *Arenaviridae* family comprising Lymphocytic choriomeningitis, Lassa fever, Argentine Hemorragic fever, Bolivian Hemorragic fever viruses; *Bornaviridae* family comprising viruses causing central nervous system diseases mainly in horses and sheep; *Reoviridae* family including rotaviruses, the most important cause of severe diarrheal illness in infants and young children worldwide, orbiviruses that can affect both humans and other mammals (bluetongue, epizootic hemorrhagic disease viruses); Retroviridae family, a large group of viruses comprising important human pathogens like human immunodeficiency virus 1 and 2 (HIV-1 and HIV-2) and human t-cell leukemia virus type 1 and 2 (HTLV 1 and 2) as well as non-human lentivirus such as Maedi/Visna viruses affecting sheep and goats, Equine infectious anemia virus affecting horses, bovine immunodeficiency virus affecting cattle, feline immunodeficiency virus affecting cats; Polyomaviridae family groups small DNA oncogenic viruses, prototype viruses are polyoma and SV40 infecting mouse and rhesus monkey respectively, (BK and JC viruses closely related to SV40 were isolated from human patients); *Papillomaviridae* family consists of a group of DNA viruses infecting higher vertebrates

including humans generating warts and condylomas. Papilloma viral infection is associated with the development of cancer in both humans and animals. Human papilloma viruses are associated with cervical cancer, vaginal cancer and skin cancer. The herpesviridae families includes subfamilies in which are classified a number of important pathogens for humans and other mammals. Suitable sources of antigens can be but are not limited to herpes simplex viruses 1 and 2, varicella-zoster virus, Epstein-Barr virus, Cytomegalovirus, human herpesviruses 6A,6B and 7, Kaposi's sarcoma-associated herpesvirus. Further suitable source of antigens are members of the Poxviridae family like Monkeypox virus, Molluscum contagiusum virus, smallpox virus; Hepatitis B virus, the prototype member of the hepadnaviridae family as well as other virus causing acute and/or chronic hepatitis like hepatitis delta virus, hepatitis E virus.

The adenoviral vectors of the present invention are also suitable for the preparation of immunogenic compositions designed to stimulate an immune response in humans or animals against protein expressed by non-viral pathogens including bacteria, fungi, parasites pathogens. For example, the vectors disclosed herein can be used to prepare vaccines against, but not limited to: *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Vibrio cholerae, Clostridium tetani, Neisseria meningitis, Corynebacterium diphteriae, Mycobacteria tuberculosis and leprae, Listeria monocytogenes,* and *Legionella pneumofila.* Examples of fungi and mammalian parasites for which it may be desirable to prepare prophylactic or therapeutic vaccines include: *Candida albicans, Aspergillus fumigatus, Histoplasma capsulatum, Plasmodium malariae, Leishmania major, Trypanosome cruzi and brucei, Schistosoma haematobium, mansoni and japonicum; Entamoeba histolytica,* and numerous species of Filaria known to be responsible for human filariasis.

Cancer typically involves the deregulation of genes that encode polypeptides which contribute to maintaining cell cycle or controlling cell proliferation (e.g., growth factors, oncogenes, receptors and tumor suppressors). The products of many of the genes implicated in cancer are expressed on the surface of a wide variety of tumor cells. A variety of tumor antigens that may be recognized by T and B lymphocytes have been identified in human and animal cancer. The vast majority of human tumor-associated antigens (TAAs) that are suitable for use in an anticancer vaccine trial are described as "self-antigens" due to the fact that in addition to being expressed on tumor cells they also are expressed on normal tissue and/or during fetal development. Immunotolerance of the target population to TAAs may explain why many cancer vaccines have proven to be ineffective.

Tumor antigens can be produced by oncogenic mutants of normal cellular genes altered proto-oncogenes or tumor suppressor genes such as Ras, p53 or Bcr-Abl protein are examples of altered cellular proteins that can stimulate T/B cell response. Tumor antigens can be normal cellular proteins that are overexpressed in tumor cells (tyrosinase, GP100, MART are normally expressed at low levels in melanocytes and overexpressed in melanoma) or aberrantly expressed in tumor cells (MAGE, BAGE,

GAGE expressed in melanomas and many carcinomas but normally expressed in the testis and placenta). Tumor antigens can be products of oncogenic viruses: papillomavirus E6 and E7 proteins expressed by cervical carcinomas; EBV EBNA-1 protein produced by EBV+ lymphomas and nasopharyngeal carcinomas; SV40 T antigen in SV40 induced experimental tumors. Oncofetal antigens are expressed to high levels on cancer cells and in normal developing (fetal) tissues but not in adult tissues. Carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) are examples of well characterized oncofetal antigens.

Recent evidence supports the existence of TAAs that are capable of eliciting an immune response, thus making this class of antigens suitable immunogens for vaccine therapy. However, as a class of antigens TAAs are notoriously poor immunogens and T cells that are highly specific for TAAs are either deleted or anergized during T-cell development. Accordingly, there is an expectation that the immune response of a tumor-bearing host to a particular TAA will be extremely weak. Because of the inherent need to break host tolerance to a target TAA experimental clinical vaccine studies are particularly focused on developing immunization strategies that will enhance TAA-specific T-cell responses. Generally, speaking an effective cancer vaccine must both overcome immunotolerance and enhance host's immune response to a level that is preventative and/or protective. Anti-tumor effects in many experimental vaccine studies have been correlated with T-cell responses to TAAs.

In an alternative embodiment, the invention contemplates an immunogenic composition (e.g., a cancer vaccine) which can be used to induce an immune response against tumor antigens. A suitable composition would contain a recombinant chimpanzee adenovirus comprising nucleic acid sequence encoding a tumor antigen and a physiologically acceptable carrier. In a particular embodiment, the coding sequence element of the cassette may encode a single immunogen, such as an immunogenic peptide sequence derived from a self-antigen, such as a tumor-associated antigen. In some embodiments, the nucleic acid sequence encoding the immunogen (i.e., the transgene) may be codon optimized for expression in a particular mammalian species. In other embodiments, the coding sequence may encode more than one immunogen, such as one or more codon optimized tumor antigens. For example, a cancer vaccine utilizing the disclosed adenoviral vectors may encode a combination of self-antigens such as: HER2/neu, CEA, Hepcam, PSA, PSMA, Telomerase, gp100, Melan-A/MART-1, Muc-1, NY-ESO-1, Survivin, Stromelysin 3, Tyrosinase, MAGE3, CML68, CML66, OY-TES-1, SSX-2, SART-1, SART-2, SART-3, NY-CO-58, NY-BR-62, hKLP2, VEGF.

Development of an effective cancer vaccine requires the identification of a strategy that will elicit antigen-specific immunity in vaccinated patients and the generation of an immune response that will persist after active immunization has ended. The success of the strategy will depend on whether a measurable immune response directed against a target antigen will correlate with protection against cancer occurrence or relapse. The effector mechanisms of both cell-mediated immunity and humoral

immunity have been show to kill tumor cells. However, data from experimental systems suggest that antigen-specific T cells represent the most powerful immunologic mechanism for the elimination of tumor cells. Recognition of tumor-specific antigens (e.g., TAAs) by effector T-cells is predicted to allow the TAA to function as a tumor-rejection antigen. Published studies suggest that stimulation of $CD8^+$ and $CD4^+$ helper T-cell responses are important for achieving optimal tumor clearance ((Greenberg, P. D. (1991) *Adv. Immunol.* 49: 281-355; Pardoll, D. M. *et al.* (1998) *Curr. Opin. Immunol.* 10: 588-94). Clinical response (i.e., efficacy) has been associated with increases in interferon γ-secreting cytotoxic T cells. The advent of assays, such as the ELISPOT assay used herein, to demonstrate the efficacy of the instant vaccine carriers, allows investigators to measure T-cell responses to vaccination regimens and thereby facilitates the development of cancer vaccines.

Cancer vaccines can be either prophylactic or therapeutic. The general assumption underlying the prophylactic use of cancer vaccines is that TAAs are extremely weak immunogens or functionally nonimmunogenic in tumor-bearing subjects. More specifically, in the field of cancer immunology, vaccines can be used as immunotherapy in patients afflicted with cancer. Accordingly, cancer vaccines can be designed to elicit an immune response that is that is directed against a TAA that is expressed by a pre-existing tumor or malignancy. Thus, in particular embodiments, therapeutic cancer vaccines are intended for use in tumor-bearing patients who have developed resistance to conventional regimens of treatment or who have a high probability of developing a recurrence following conventional treatment.

The high immunogenicity of adenoviruses, make adenoviral vectors particularly good candidates for use in the context of a vaccine carrier designed to break host tolerance to a self-antigen. The phenomenon of epitope or determinant spreading, which was first described in autoimmune diseases, has been associated with both MHC class I- and MHC class II-restricted responses and correlated to the development of HER-2/neu protein-specific T-cell immunity. Epitope spreading represents the generation of an immune response to a particular portion of an immunogenic protein followed by the natural spread of immunity to other antigenic determinants present on the same protein. For example, Disis *et al.* observed epitope spreading in 84% of patients afflicted with HER-2/neu overexpressing malignancies who were administered vaccines comprising peptides derived from potential T-helper epitopes of the HER-2 protein mixed with granulocyte-macrophage colony stimulating factor (*J. Clin. Oncol.* (2002) 20(11): 2624-2632). Importantly, epitope spreading was correlated with the generation of a HER-2/neu protein domain response and suggests that immunization effectively circumvented immunologic tolerance.

TAAs that are suitable for use in the disclosed adenoviral vectors and methods as a target for a cancer vaccine should possess a number of characteristics. For example, a target TAA must have a favorable expression profile, meaning that it should be preferentially expressed or overexpressed in the

tumor or malignant tissue as compared with normal tissue. In addition, because TAAs that play a role in tumorigenesis are more likely to be retained during the different stages of cancer progression, a suitable target TAA should also preserved throughout tumor progression and metastases. Suitable target TAAs should also be expressed homogenously within the tumor. Third, suitable target TAAs must not be subject to absolute immunologic tolerance. More specifically, there should be some evidence that T cells which can both recognize and respond to the TAA of interest have not been entirely deleted from the host's T-cell repertoire (Berinstein, N. L., *J. Clin. Oncol.* 29(8): 2197 (2002).

Carcinoembryonic antigen (CEA) has many characteristics which make it an attractive TAA for use as a target antigen for an anticancer vaccine. It is a member of the Ig superfamily which is characterized by a favorable expression pattern. It is expressed in more than 50% of all human cancers and has been implicated in the tumorigenesis process, which suggests that its expression may be selected and conserved throughout cancer progression. In addition, it has been established that immunologic tolerance to CEA is not absolute. Published studies establish that human T cells can recognize, become activated to, and lyse cancer cells that express CEA (Berinstein, N. L., J. *Clin. Oncol.* 29(8): 2197 (2002). For example, the immunization of patients with recombinant vaccinia virus expressing CEA, combined with subsequent peptide-based in vitro stimulations, generated CD8+ MHC-restricted CTLs capable of lysing autologous tumors (Tsang, K. Y. *et al. J. Natl. Cancer Inst.*, (1995) 87:982-990). Alternatively, immunization of colorectal carcinoma patients after surgery with recombinant CEA was reported to induce weak antibody and cellular responses to recombinant CEA (Samanci, A., *et al.* (1998) *Cancer Immunol. Immunother.* 47: 131-142.) Further, the administration of anti-CEA anti-idiotypic antibody to patients diagnosed with colorectal cancer generated anti-CEA antibodies and idiotype-specific T-cell proliferation (Foon, L, A. *et al.* (1995) *J. Clin. Invest..*, 96: 334-342). The literature also indicates that tolerance to CEA in cancer patients can be overcome with several different vaccination approaches (i.e., vaccination with recombinant CEA or recombinant orthopox or avipox-CEA viruses, administration of anti-idiotype antibodies, pulsing dendritic cells with CEA agonist epitopes).

CEA is an oncofetal glycoprotein that is expressed in normal fetal colon and to a much lesser extent in normal colonic mucosa. It is also overexpressed in the vast majority of adenocarcinomas, particularly those of the colon, pancreas, breast, lung, rectum and stomach. Many colorectal cancers and some carcinomas produce high levels of CEA that are measurable in sera, which makes it one of the most widely used serological markers of malignancy, especially in patients with colorectal cancer.

A second TAA which provides a suitable immunogen for use in the compositions and methods of the invention is product of the HER2/erb-2 (also called neu) proto-oncogene. Like, CEA, HER2/neu has a favorable expression pattern and is not subject to absolute tolerance. More specifically, low levels of expression of the HER2/neu transcript, and the 185 kD polypeptide product, are detected in normal adult epithelial cells of various tissues, including the skin and breast, and tissues of the

gastrointestinal, reproductive, and urinary tracts; higher levels of expression are detected in the corresponding fetal tissues during embryonic development (Press *et al.*, *Oncogene* 5: 953-962 (1990). Several lines of evidence suggest a link between the amplification of HER-2 and neoplastic transformation in human breast, lung, prostate, ovarian, endometrial and colorectal tumors (Disis and Cheever, *Adv. Cancer Research 71*: 343-371(1997). Generally speaking, overexpression of HER2/neu correlates with a poor prognosis and a higher relapse rate for cancer patients (Slamon *et al.*, *Science* 244: 707-712 (1989). Thus, a vaccine specific for the HER-2/neu protein could have wide application and utility in the prevention of disease recurrence in many different human malignancies.

HER2/neu encodes a transmembrane glycoprotein possessing intrinsic tyrosine kinase activity and displaying extensive homology to the epidermal growth factor (EGF) receptor (Akiyama, T *et al.*, (1986) Science 232: 1644-1646). One of the first clinical studies which utilized HER2 as target for cancer immunotherapy employed the HER-2-specific monoclonal antibody Herceptin for the treatment of breast cancer (Goldenberg MM (1999) *Clin. Ther.* 21: 309-318). This led to subsequent efforts which focused on the use of HER-2 as a target for the T-cell arm of the immune system to elicit effective antitumor responses, including the use of recombinant fusion proteins comprising HER-2 domains to activate autologous antigen presenting cells. Published reports establish that numerous cancer patients afflicted with neu-expressing mammary and ovarian cancers mount immune responses (e.g., produce antigen-specific antibody and T-cells) against the protein product of the HER2/neu oncogene.

Assembly of the recombinant adenoviral sequences, transgene and other vector elements into various intermediate plasmids and shuttle vectors, and the use of the plasmids and vectors to produce a recombinant viral particle are all achieved using conventional techniques as described in standard textbooks that are well known to those of skill in the art (Sambrook *et al*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). Such techniques include, but are not limited to conventional cDNA cloning techniques, use of overlapping oligonucleotde sequences derived from the adenoviral genome, homologous recombination, polymerase chain reaction, standard transfection techniques, plaquing of viruses in agar overlay and other related methodologies.

To assist in preparation of polynucleotides in prokaryotic cells, a plasmid version of the adenovirus vector is often prepared (adenovirus pre-plasmid). The adenovirus pre-plasmid contains an adenoviral portion and a plasmid portion. The adenoviral portion is essentially the same as the adenoviral portion contained in the adenoviral vectors of the invention (containing adenoviral sequences with non-functional or deleted E1 and optionally E3 regions) and an immunogen expression cassette, flanked by convenient restriction sites.

The plasmid portion of the adenovirus pre-plasmid often contains an antibiotic resistance marker under transcriptional control of a prokaryotic promoter so that expression of the antibiotic does not occur in eukaryotic cells. Ampicillin resistance genes, neomycin resistance genes and other

pharmaceutically acceptable antibiotic resistance markers may be used. To aid in the high level production of the polynucleotide by fermentation in prokaryotic organisms, it is advantageous for the adenovirus pre-plasmid to contain a prokaryotic origin of replication and be of high copy number. A number of commercially available prokaryotic cloning vectors provide these benefits. It is desirable to remove non-essential DNA sequences. It is also desirable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of integration of polynucleotide vaccine sequences into the recipients' genome. Tissue-specific promoters or enhancers may be used whenever it is desirable to limit expression of the polynucleotide to a particular tissue type.

Adenovirus pre-plasmids (plasmids comprising the genome of the replication-defective adenovirus with desired deletions and insertions) can be generated by homologous recombination using adenovirus backbones DNA and an appropriate shuttle vector (designed to target-in specific deletions and incorporate desired restriction sites into the resultant plasmid). Shuttle vectors of use in this process can be generated using general methods widely understood and appreciated in the art, *e.g.*, PCR of the adenoviral terminal ends taking into account the desired deletions, and the sequential cloning of the respective segments into an appropriate cloning plasmid. The adenoviral pre-plasmid can then be digested and transfected into the complementing cell line via calcium phosphate co-precipitation or other suitable means. Virus replication and amplification then occurs, a phenomenon made evident by notable cytopathic effect. Infected cells and media are then harvested after viral replication is complete (generally, 7-10 days post-transfection).

Generally speaking, following the construction and assembly of the desired adenovirus pre-plasmids, adenovirus pre-plasmids are rescued into virus by transfecting an adenoviral E1-expressing human cell line. Complementation between the packaging cell line and the viral genes of the vector permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the production of recombinant adenoviruses. The resulting viruses may be isolated and purified by any of a variety of methods known to those of skill in the art for use in the methods of the invention.

It will be readily apparent to those of skill in the art that when one or more selected deletions of chimpanzee adenoviral genes are introduced into a viral vector, the function of the deleted gene product can be supplied during the production process by sequences present in the production cell line. Thus, the function of the manipulated genes can be provided by a permanently transformed cell line that is characterized by some or all of the adenoviral functions which are required for packaging but which are not functional in the vector (e.g., any of E1A, E1B, E2A, E2B E4). Alternatively, the requisite adenoviral functions can be provided to a suitable packaging cell line by infecting or transiently transfecting a suitable cell with a construct comprising the requisite gene to provide the function.

Accordingly, the present invention also provides a method of producing chimpanzee adenoviral vectors in E1-expressing human cell lines. More specifically, the disclosed vectors can be propagated in an E1 complementing cell lines, including the known cell lines 293 and PER.C6™. Both these cell lines express the adenoviral E1 gene product. PER.C6™ is described in WO 97/00326, published January 3, 1997, which is hereby incorporated by reference. It is a primary human retinoblast cell line transduced with an E1 gene segment that complements the production of replication deficient first generation adenoviruses, but is designed to prevent generation of replication competent adenovirus by homologous recombination. 293 cells are described in Graham *et al* (1977) *J. Gen. Virol* 36:59-72, which is also hereby incorporated by reference. One of skill in the art will recognize the term "first generation adenovirus" refers to a replication deficient adenovirus which has either a non-functional or deleted E1 region, and optionally a non-functional or deleted E3 region.

Batches of replication-defective adenoviral vectors that are intended for use as a vaccine composition in a clinical trial should be proven to be free of RCA (Fallaux, F.J. *et al* (1998) *Human Gene Therapy*, 9:1909-1917). In practice, this is a labor intensive process which requires establishing and utilizing an expensive screening program. One of skill in the art will acknowledge that a high frequency of RCA generation not only results in a high failure rate for the batches produced, but also severely limits scale-up efforts. Elimination of sequence homology between the nucleotide sequence of the vector and the adenoviral sequences present in the genome of the helper production/packaging cell line should eliminate the possibility of producing batches of vector that are contaminated with RCAs produced by homologous recombination.

Typically, recombinant replication-defective adenoviral vectors are propagated in cell lines that provide E1 gene products *in trans*. Supplementation of the essential E1 gene products *in* trans is very effective when the vectors are from the same or a very similar serotype. For example, it is well-known that E1-deleted (i.e. ΔE1) group C serotype (Ad2 and Ad5) vectors, can be propagated in 293 or PER.C6 cells which contain and express the Ad5 E1 region. However, it has been observed that Ad5 E1 sequences present in the 293 and PER.C6 production cells may not always fully complement the replication of non-group C serotypes. Accordingly, E1-deleted serotypes outside of subgroup C, for example those from subgroups A, B, D, E, and F may replicate with a lower efficiency respect to the corresponding wt virus or may not replicate at all in 293 or PER.C6 cells. This may be due to the inability of the Ad5 (group C) E1B 55K gene product to establish a functional interaction with the E4 orf6 gene product of the non-group C serotypes.

The decrease in replication efficiency in cells expressing Ad5 E1 is variable considering vectors of different subgroups. While ΔE1 vectors deriving from subgroup D and E adenovirus can be rescued and propagated in 293 and Per.C6™ cells with variable efficiency, the propagation ΔE1 vectors of subgroup B is completely impaired (Vogels R, *et. al.* (2003) Aug. Replication-deficient human

adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol.* 77 (15):8263-71).

Although the interaction between Ad5 E1b 55k and vector-expressing E4 orf6 protein is conserved within members of the same subgroup, it may be not sufficiently stable when E4 orf6 protein of a non-C serotype is expressed. This inefficient or unstable formation of E1B-55K/E4-orf6 complex lead to an absent of reduced propagation of the ΔE1 vector. Accordingly, it has been empirically determined that in order to successfully and efficiently rescue recombinant adenovirus of groupB serotypes, a cell line expressing the E1 region of the serotype of interest may need to be generated. In cells expressing Ad5E1 like 293 or Per.C6™, the expression can be limited to E1b 55K protein. Alternatively, a suitable Ad5E1-expressing cell lines could be modified to express the entire Ad5 E4 region (or E4 orf6 only) in addition to Ad5E1. The generation of cell lines expressing both Ad5 E1 and orf6 are useful in complementing alternative adenovirus serotypes; *see, e.g.,* Abrahamsen *et al.*, 1997 *J. Virol.* 8946-8951. The incorporation of E4 (orf6) into Ad5 complementing cell lines, is known, as is the generation of serotype-specific cell lines providing a serotype-specific E1 gene product(s) *in trans.* Alternatively, the efficiency of non-group C vector propagation may be improved by modification of the viral backbone by substituting the native E4 region with Ad5 orf6. Similar results can be achieved by substituting the only the native orf6 with orf6 deriving from Ad5 or other subgroup C viruses (Ad1, Ad2, Ad6). U.S. Patent No. 5,849,561 discloses complementation of an E1-deleted non-group C adenovirus vector in an Ad5-E1 complementing cell line which also expresses portions of the Ad5-E4 gene.

U.S. Patent No. 6,127,175, issued to Vigne, *et al.*, discloses a stably transfected mammalian cell line which expresses a portion of the E4 region of adenovirus, preferably orf6/orf6/7. Such a cell line is useful for complementation of recombinant Ad genomes deficient in the E4 region.

Compositions, including vaccine compositions, comprising the disclosed adenoviral vectors are an important aspect of the present invention. These compositions can be administered to mammalian hosts, preferably human hosts, in either a prophylactic or therapeutic setting. Potential hosts/vaccinees include but are not limited to primates and especially humans and non-human primates, and include any non-human mammal of commercial or domestic veterinary importance. Compositions comprising recombinant chimpanzee adenoviral vectors may be administered alone or in combination with other viral- or non-viral-based DNA/protein vaccines. They also may be administered as part of a broader treatment regimen.

In a particular embodiment of the invention, the disclosed vectors may be used in an immunization protocol designed to break host tolerance to a self-antigen or a tumor-associated antigen. The identification of a number of TAA has enabled the development of active vaccination approaches for the therapy of cancer. Both cell surface antigens and intracellular antigens that are processed and presented provide useful targets. Generally speaking, the disclosed method of breaking host tolerance to a

self-antigen comprises: (a) stimulating an antigen-specific response to a self-antigen by administering a first vaccine composition comprising a first ChAd vector or a plasmid vector carrying a nucleotide sequence encoding the self-antigen against which an antigen-specific immune response is desired, and (b) sustaining and expanding the immune response of (a) by administering a second vaccine composition comprising a recombinant ChAd vector of a different serotype containing at least a functional deletion of its genomic E1 gene, and in the site of the E1 gene, a sequence comprising a promoter capable of directing the expression of DNA encoding the same self-antigen delivered in the priming step, whereby the host mounts an immune response which has the effect of breaking tolerance to the self-antigen.

Accordingly, a skilled artisan can utilize this disclosure to design several different immunization protocols that may be suitable for use to break host tolerance. For example, it may be possible to utilize a protocol in which the first, or priming immunization comprises plasmid DNA which encodes a particular self-antigen, such as a TAA, and any subsequent immunizations comprise a ChAd vector. Plasmid DNA sequences comprising nucleotide sequences that encode self-antigens, may be delivered intramuscularly, with or without electrostimulation, in one or more injections. For example, an immunization protocol based on multiple (e.g., 3 or 4 or 5) intramuscular injections of plasmid DNA encoding a TAA via electroporation followed by one or more intramuscular injections of a ChAd vector comprising a transgene encoding the same TAA is encompassed by the general method disclosed and claimed herein.

Alternatively, a suitable protocol to break tolerance could involve one or more priming immunizations with a ChAd or hAd vector comprising a transgene encoding a self antigen, followed by one or more boosting immunizations with either the same, or a different ChAd vector that is know to be non cross-reactive with the vector used for the priming immunization(s). For example, an immunization protocol using ChAd3 for priming and ChAd6 for boosting, or ChAd3 for priming followed by ChAd6 and ChAd9 for boosting could be used to break host tolerance. In particular embodiments, the invention contemplates the use of self-antigens comprising at least one tumor associated antigen selected from the group consisting of: HER2/neu, CEA, EpCAM, PSA, PSMA, Telomerase, gp100, Melan-A/MART-1, Muc-1, NY-ESO-1, Survivin, Stromelysin 3, Tyrosinase, MAGE3, CML68, CML66, OY-TES-1, SSX-2, SART-1, SART-2, SART-3, NY-CO-58, NY–BR-62, hKLP2, VEGF. In a particular embodiment, the invention provides a method for inducing an immune response (e.g., humoral or cell-mediated) to a tumor-associated antigen which is specific for a selected malignancy by delivering a recombinant chimpanzee adenovirus encoding the TAA to a mammal afflicted with cancer. In a preferred embodiment of this aspect of the invention the elicited immune response constitutes an immune response characterized by the production of antigen-specific CD4+ and CD8+ T cells.

The immunogenic compositions of the invention can be administered to mammalian hosts, preferably human hosts, in either a prophylactic or therapeutic setting. Potential hosts/vaccinees include

but are not limited to primates and especially humans and non-human primates, and include any non-human mammal of commercial or domestic veterinary importance. Compositions comprising recombinant chimpanzee adenoviral vectors may be administered alone or in combination with other viral- or non-viral-based DNA/protein vaccines. They also may be administered as part of a broader treatment regimen. Suitable compositions, for use in the methods of the invention may comprise the recombinant viral vectors of the invention in combination with physiologically acceptable components, such as buffer, normal saline or phosphate buffered saline, sucrose, other salts and polysorbate. It does not cause tissue irritation upon intramuscular injection. It is preferably frozen until use. Optionally, a vaccine composition of the invention may be formulated to contain other components, such as but not limited to, an adjuvant, a stabilizer, a pH adjusting agent, or a preservative. Such components are well known to those of skill in the art.

It is envisioned that the recombinant chimpanzee adenoviruses of the invention will be administered to human or veterinary hosts in an "effective amount," that is an amount of recombinant virus which is effective in a chosen route of administration to transduce host cells and provide sufficient levels of expression of the transgene to invoke an immune response which confers a therapeutic benefit or protective immunity to the recipient/vaccine.

The amount of viral particles in the vaccine composition to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of $1x10^7$ to $1x10^{12}$ particles (i.e., $1x\ 10^7$, $2x10^7$, $3x10^7$, $5x10^7$, $1x10^8$, $2x10^8$, $3x10^8$, $5x10^8$ or $1x10^9$, $2x10^9$, $3x10^9$, $5x10^9$) and preferably about $1x10^{10}$ to $1x10^{11}$ particles is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated.

The recombinant chimpanzee adenoviral vectors of the present invention may be administered alone, as part of a mixed modality prime/boost vaccination regimen or in a vaccination regimen based on combination of multiple injections of different vector serotypes. Typically, a priming dose(s) comprising at least one immunogen is administered to a mammalian host in need of an effective immune response to a particular pathogen or self-antigen. This dose effectively primes the immune response so that, upon subsequent identification of the antigen(s), the host is capable of immediately mounting an enhanced or boosted immune response to the immunogen. A mixed modality vaccination scheme which utilized alternative formulations for the priming and boosting can result in an enhanced immune response. Prime-boost administrations typically involve priming the subject (by viral vector, plasmid, protein, *etc.*) at least one time, allowing a predetermined length of time to pass, and then boosting (by viral vector, plasmid, protein, *etc.*). Multiple immunizations, typically 1-4, are usually employed, although more may be used. The length of time between priming and boost may typically vary

from about four months to a year, albeit other time frames may be used as one of ordinary skill in the art will appreciate. Multiple injection of each vector can be administered within approximately a 2 weeks time frame, before neutralizing immunity becomes evident.

In some embodiments of this invention, a vaccine is given more than one administration of adenovirus vaccine vector, and it may be given in a regiment accompanied by the administration of a plasmid vaccine. Suitable plasmid vaccines for use in combination with the vectors disclosed herein comprise a plasmid encoding at least one immunogen against which a primed or boosted immune response is desired, in combination with a heterologous promoter, which is capable of directing expression of the nucleic acid sequences encoding the immunogen(s), operably linked to the immunogen coding sequence, and a transcription terminator sequence.

For example, a dosing regimen which utilizes multiple injection of different serotypes of recombinant replication-defective chimpanzee adenoviral vectors can be used. Alternatively, an individual may be given a first dose (i.e., a priming dose) of a plasmid vaccine, and a second dose (i.e., a boosting dose) which comprises a replication-defective recombinant chimpanzee adenoviral vector which comprises a coding sequence for the same immunogen that was delivered in the plasmid vaccine. Alternatively, the individual may be given a first dose of a human adenovirus vaccine vector encoding at least one immunogen, followed by a second dose comprising a replication-defective recombinant chimpanzee adenoviral vector disclosed herein, which comprises a coding sequence for the same immunogen that was delivered in the priming dose. In a second alternative embodiment a vaccine composition comprising a vector of the invention may be administered first, followed by the administration of a plasmid vaccine. In any of these embodiments, an individual may be given multiple doses of the same immunogen in either viral vector or plasmid form. There may be a predetermined minimum amount of time separating the administrations.

In addition to a single protein or antigen of interest being delivered by the recombinant, replication-defective chimpanzee adenovirus vectors of the present invention, two or more proteins or antigens can be delivered either via separate vehicles or delivered *via* the same vehicle. Multiple genes/functional equivalents may be ligated into a proper shuttle plasmid for generation of a adenovirus pre-plasmid comprising multiple open reading frames. Open reading frames for the multiple genes/functional equivalents can be operatively linked to distinct promoters and transcription termination sequences.

As shown herein, suitable immunization regimens can employ different adenoviral serotypes. One example of such a protocol would be a priming dose(s) comprising a recombinant adenoviral vector of a first serotype, for example a ChAd3 or ChAd6 followed by a boosting dose comprising a recombinant chimpanzee adenoviral vector of a second serotype. In an alternative embodiment, the priming dose can comprise a mixture of separate adenoviral vehicles each comprising a

gene encoding for a different protein/antigen. In such a case, the boosting dose would also comprise a mixture of vectors each comprising a gene encoding a separate protein/antigen, provided that the boosting dose(s) administers recombinant viral vectors comprising genetic material encoding for the same or similar set of antigens that were delivered in the priming dose(s). These multiple gene/vector administration modalities can further be combined. It is further within the scope of the present invention to embark on combined modality regimes which include multiple but distinct components from a specific antigen.

Use of recombinant vectors derived from chimpanzee adenoviruses that are not neutralized by preexisting immunity directed against the viral elements of human vector offers an alternative to the use of human Ad vectors as vaccine carriers. Because adenoviruses are highly immunogenicity, adenoviral vectors are particularly good candidates for use in the context of a vaccine carrier designed to break host tolerance to a self-antigen. Furthermore, the ability to propagate the chimp viruses in human cells, particularly in the Per.C6™ cell line, with an efficiency comparable to human viruses, offers considerable advantages both from a regulatory point of view and for the large scale production of therapeutics or vaccines. Accordingly, the instant invention provides a collection of chimpanzee adenoviral sequences, vectors and plasmids that allow the preparation of recombinant virus which may be used, alone or in combination, as a vaccine carrier for genetic vaccination.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

Example 1: Isolation, Cloning, Sequencing And Characterization Of ChAds

Chimpanzee Adenovirus Isolation

Stool specimens were collected in viral transport medium (VTM; Microtest M4-R Multi-Microbe Transport Medium, Remel Inc.) then frozen or frozen directly at –70°C at NIRC (New Iberia Research Center 4401 W. Admiral Doyle Drive New Iberia, LA 70560). The specimens were kept frozen at < -70°C until they were processed for inoculation into cell cultures. At that time, the specimens were thawed and then vortexed in excess of chilled viral transport medium. After the specimens had

dissociated into suspensions, they were centrifuged for 10 min at 1500-1800 rpm. The supernatants were filtered through 0.8 and 0.2 μm syringe filters in series and then the filtered material was inoculated into cell cultures (200-250 μL into shell vials and 250-300 μL into tube cultures). Each processed specimen was inoculated into tube cultures and shell vial cultures seeded with 293 cells or A549 cells.

Control (positive and negative) cultures were prepared each time a set of samples was inoculated. Once all of the shell vials in a set-up had been inoculated, they were centrifuged at room temperature for 60 ± 10 min at 2000 rpm (900 x g). The vials were removed from the centrifuge immediately after the rotor stopped spinning to prevent heat damage in the cultures. After centrifugation, the inocula were aspirated from the shell vials, using a fresh sterile Pasteur pipette in each vial to prevent cross-contamination. The cultures were washed three times using 1.0-mL fresh culture medium for each wash. Fresh medium (1.0 mL) was pipetted into each vial after the third wash and the shell vials were placed in an incubator at 35-37°C for three to four days (approx. 96 hr).

At the end of the culture period, the supernatants were aspirated from the cultures and the cell layer in each vial was washed twice with Immunofluorescence Assay (IFA) Buffer using approximately 1.0 mL buffer with each wash. The cells were fixed by adding 1.0 mL refrigerated acetone to each vial (10 min at 2-8°C. Acetone-cleaned slides were labeled with the specimen identification number(s) associated with the shell vial coverslips. The shell vial coverslips were processed for fluorescence labeling of Adenovirus-infected cells using a primary mouse anti-adenovirus antibody [MAB8052, Chemicon]. The slides are evaluated with the aid of a fluorescence microscope. Each preparation was scanned using the 10X objective noting the extent of immunofluorescence coverage across the well (1+ to 4+). The presence or absence of specific immunofluorescence was confirmed using the 40X objective. Tube cultures were inoculated in the same sequence as described for the shell vials (e.g., negative control first, followed by clinical specimens and positive controls). The inocula were allowed to adsorb for 60-120 min at 36-38°C. After the adsorption period, the specimens/controls were aspirated from the tubes and replaced by fresh culture medium.

Three to four days post-inoculation, and once a week thereafter, the media was aspirated from the culture tubes and replaced with 1.5 mL fresh media. Culture tubes were visually monitored for CPE at least every other day for at least 21 days after inoculation. Cultures inoculated with chimp specimens were compared against the controls and rated by observing the CPE extent. Cultures showing no CPE were passed to fresh tube cultures after 14 days; culture tubes that were negative for CPE after 21 days were considered negative. Culture tubes with 3-4+ CPE were vortexed for 10 seconds. The cells were scraped from the wall of the tube using a sterile 1.0 mL serological pipette and suspended in the culture supernatant. After labeling a 5 mL snap cap tube with the specimen identification number and date and stored at –70°C. 500 μL of the cell suspension was transferred from the culture tube into the snap cap tube and stored for up to one day at 2-8°C until it was processed using an indirect

immunofluorescent antibody technique to detect adenovirus (equivalent to procedure for staining shell vials).

Chimpanzee Adenovirus Amplification

Wild type chimp adenoviruses CV32, CV33, CV23 and CV68 purchased from the ATCC (ATCC Accession Numbers: CV32, VR-592; CV-33, VR-593;) or from Esoterix Inc. Austin, Texas and original isolates were propagated as follows by using the human E1-expressing cell line PER.C6™ or 293. Briefly, cells were cultivated in Dulbecco's Modified Eagles Medium (DMEM; GibcoBRL, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS GibcoBRL, Life Technologies), 1% Penicillin-Streptomycin, 2mM Glutamine and 10mM $MgCl_2$ (Per.C6™). Adenovirus infection was carried out in DMEM supplemented with 5% Horse Serum (GibcoBRL, Life Technologies). Infected cells and medium were collected when 100% of the cells exhibited virus-induced cytopathic effect (CPE) and lysed by three cycles of freezing and thawing.

All wild type chimp adenoviral (CV) stocks were cloned by infecting 293 cells seeded in 96-well plates, after the first passage of amplification. The virus cloning was performed by limiting dilution of the cell lysate obtained at the first passage of the virus amplification. Five isolated clones were picked up and serially propagated. After 3-4 serial passages of amplification, a large-scale preparation of adenovirus was performed on cells planted on 5 two-layer cell-factories (NUNC) (200 millions of cells/cell factory). Purified viral particles were obtained from cell lysate by two ultra-centrifugation steps on cesium chloride density gradients.

Sequencing of Viral Genomic DNA

Genomic DNA was isolated from 3 X 10^{12} pp of purified virus preparation by digestion with Proteinase K (0.5 mg/ml) in 1% SDS-TEN (2 hrs at 55°C). After a Phenol-Chloroform extraction and Ethanol precipitation, the genomic DNA was resuspended in water and submitted for genomic sequencing.

For full length Ad genome sequencing, the purified viral DNA was nebulized to produce randomly sheared fragments. The DNA fragments were blunt-ended with the klenow fragment of E.coli DNA polymerase and polynucleotide kinase. The blunt end fragment were run on a low melting point agarose gel to purify the fragments in the size range of 1-3 Kb and cloned into the SmaI site of pUC19 vector to create a shotgun library. The ligations were used to transform competent XL1-Blue MRF'. Positive colonies were identified by white/blue screening on LB agar containing X-gal and IPTG. Three to four 96-well block of plasmid DNA were isolated from the library and sequenced with pUC forward and reverse primers. All sequencing reads were screened for quality and vector sequence using the Phred-Phrap software package. The reads that passed the screening were assembled into

contigs. Primers were designed to directly sequence the adenoviral DNA for closing the gaps and determine the DNA sequence of both ends.

Complete viral genome sequencing was obtained for selected viruses including ChAd3 (SEQ ID NO: 1), ChAd6 (SEQ ID NO: 2), CV32 (SEQ ID NO: 3), CV33 (SEQ ID NO: 4), and CV23 (SEQ ID NO: 5). Table 1 provides data summarizing the percentage of identity between the nucleotide sequences of ChAd3, ChAd6, Pan5 (CV23), Pan6 (CV32), Pan7 (CV33), C1 and C68 adenoviral genomes. Alignments were calculated using the ALIGN program as part of the FASTA package version 2 (William R. Penson, University of Virginia; Myers & Miller, CABIOS 1989, 4:11 -17).

Table 1. Percentage of Nucleotide Sequence Identity Between Chimpanzee Adenovirus Genomes

	ChAd3	ChAd6	Pan5	Pan6	Pan7	C1	C68
ChAd3	100	68.1	68.5	68.2	68.3	64.2	68.0
ChAd6		100	95.5	94.5	95.5	73.6	91.4
Pan5			100	94.9	96.7	73.9	92.7
Pan6				100	95.1	73.6	91.3
Pan7					100	73.8	93.0
C1						100	74.3
C68							100

To characterize the new adenoviral isolates (e.g., ChAd20, ChAd4, ChAd5, ChAd7, ChAd8, ChAd9, ChAd10, ChAd11, ChAd16, ChAd17, ChAd19, ChAd22, ChAd24, ChAd26, ChAd30, ChAd31, ChAd37, ChAd38, ChAd44, ChAd63 and ChAd82) the nucleotide sequence of the hexon and fiber genes were also determined by primer walking. Fiber gene: SEQ ID NOS: 6- 15: (SEQ ID NO: 6, ChAd20); (SEQ ID NO: 7, ChAd4); SEQ ID NO: 8, ChAd5); SEQ ID NO: 9, ChAd7); SEQ ID NO: 10, ChAd9); SEQ ID NO: 11, ChAd10); SEQ ID NO: 12, ChAd11); SEQ ID NO: 13, ChAd16) SEQ ID NO: 14, ChAd17), SEQ ID NO: 15, ChAd19), and (SEQ ID NO: 58, ChAd8), (SEQ ID NO: 60, ChAd22), (SEQ ID NO: 62, ChAd24), (SEQ ID NO: 64, ChAd26), (SEQ ID NO: 66, ChAd30), (SEQ ID NO: 68, ChAd31), (SEQ ID NO: 70, ChAd37), (SEQ ID NO: 72, ChAd38), (SEQ ID NO: 74, ChAd44), (SEQ ID NO: 76, ChAd63) and (SEQ ID NO: 78, ChAd82). Figures 20A-20G provide a comparison of the amino acid sequences of the fiber proteins of the ChAd isolates disclosed and claimed herein.

The hexon gene sequences are set forth in SEQ ID NOS: 16-25: (SEQ ID NO: 16, ChAd20); SEQ ID NO: 17, ChAd4); SEQ ID NO: 18, ChAd5); SEQ ID NO: 19, ChAd7); SEQ ID NO: 20, ChAd9); SEQ ID NO: 21, ChAd10); SEQ ID NO: 22, ChAd11); SEQ ID NO: 23, ChAd16); SEQ ID NO: 24, ChAd17) SEQ ID NO: 25, ChAd19), (SEQ ID NO: 97, ChAd8), (SEQ ID NO: 99, ChAd22),

(SEQ ID NO:101, ChAd24), (SEQ ID NO: 103, ChAd26), (SEQ ID NO: 105, ChAd30), (SEQ ID NO: 107, ChAd31), (SEQ ID NO: 109, ChAd37), (SEQ ID NO: 111, ChAd38), (SEQ ID NO: 113, ChAd44), (SEQ ID NO: 115, ChAd63) and (SEQ ID NO: 117, ChAd82). Figures 31A-31J provide a comparison of the amino acid sequences of the hexon proteins of the ChAd isolates disclosed and claimed herein.

Chimpanzee Adenovirus Classification

Classification of the different chimp adenoviral strains follows the already proposed classification of human adenovirus serotypes into 6 subgroups (Horowitz, MS (1990) Adenoviridae and their replication. In Virology B.N. Fields and D.M. Knipe, eds (Raven Press, New York) pp.1679-1740) and it was obtained by amino acid and nucleotide sequence alignment by using Align X program (Informax, Inc).

An initial classification of the new isolates was obtained by looking at the restriction pattern of the viral genome with different restriction endonucleases and by sequence analysis of the hypervariable region 7 (HVR7) of the hexon gene. To this end two primers were designed on the highly conserved regions flanking HVR7: TGTCCTACCARCTCTTGCTTGA (SEQ ID NO. 45) and GTGGAARGGCACGTAGCG (SEQ ID NO. 46). The HVR7 was amplified by PCR using purified viral DNA or crude 293 lysate as template and then sequenced. Based on HVR7 sequence analysis we classified the new isolated viruses into the subgroups (A-F) proposed for human Ad viruses (Horowitz, MS (1990) Adenoviridae and their replication. In Virology B.N. Fields and D.M. Knipe, eds (raven Press, New York) pp.1679-1740).

The phylogenetic tree presented in Figure 35 was obtained by alignment of human and chimp adenovirus hexon amino acid sequences. The results are consistent with the initial classification based on nucleotide sequence alignment limited to hexon HVR7 by using Align X program (Informax, Inc). The tree was deduced from a multiple sequence alignment of full-length hexon peptide sequences using a PAUPSEARCH (Wisconsin Package Version 10.3, Accelrys Inc.) and visualized and manipulated with TREEVIEW. Bootstrap confidence analysis was performed using the PAUPSEARCH program as implemented in the Wisconsin Package. For each of the alignments the program was run on 1000 replicates using "Heuristic Search" as search criterion and Maximum Parsimony as the optimality criterion and confidence values reported were taken from a 50% majority-rule consensus.

Example 2: ChAd Shuttle Vector and Expression Vector Construction and Rescue

Vector Construction and Rescue

Genomic viral DNA was cloned into a standard plasmid vector by homologous recombination with an appropriate shuttle vector containing viral DNA sequences derived from both left and right end of viral genome (Figure 2). As described more fully below, the sequence homology

observed between viruses classified in the same serotype subgroup was exploited to develop group-specific shuttle vectors. Genomic viral DNA of Chimp adenovirus classified into subgroup D and E resulted to be sufficiently homologous to allow the construction of a common shuttle vector in order to clone viruses belonging to both subgroups.

Construction of a Subgroup D/E Shuttle Vector

The ChAd6 viral genome was fully sequenced (SEQ ID NO: 2) and the information obtained was used to construct a shuttle vector to facilitate cloning by homologous recombination of subgroup D and E chimpanzee adenovirus.

Construction of the ChAd6 shuttle vector, referred to herein as pARS ChAd6-3 is described in Figure 1. Figure 32 provides a list of the oligonucleotide sequences (SEQ ID NOS: 26-40 and SEQ ID NOS: 45-46) used in the cloning experiments described herein. Briefly, 457 bp deriving from the left end of ChAd6 DNA were amplified by PCR with the oligonucleotides 5'-ATGGAATTCGTTTAAACCATCATCAATAATATACCTC-3 (SEQ ID NO: 27) and 5'-CGCTGGCACTCAAGAGTGGCCTC-3' (SEQ ID NO: 28) digested with EcoRI and SnaBI and cloned into pNEBAd35-2 cut EcoRI-SnaBI, generating pNEBChAd6-LI. The right ChAd6 ITR (bp 36222 to bp 36648) was amplified by PCR using the oligonucleotides: 5'- ATGAAGCTTGTTTAAACCCAT CATCAATAATATACCT-3 '(SEQ ID NO: 29) and 5'- ATCTAGACAGCGTCCATAGCTTACCG-3'(SEQ ID NO: 30) digested with restriction enzymes HindIII and XbaI and cloned into pNEBChAd6-LI HindIII-XbaI digested thus generating pNEBChAd6-RLI. Finally, the DNA fragment corresponding to nucleotides 3426-3813 of the ChAd6 genomic DNA sequence was amplified with the oligonucleotides: 5' ATGCTACGTAGCGATCGCGTGAGTAGTGTTTGGGGGTGGGTGGG-3' (SEQ ID NO: 31) and 5'-TAGGCGCGCCGCTTCTCCTCGTTCAGGCTGGCG-3' (SEQ ID NO: 32), digested with SnaBI and AscI then ligated with SnaBI-AscI digested pNEBChAd6-RLI thus generating pNEBChAd6-RLIdE1.

To improve the efficiency of recombination and plasmid propagation in DH5a *E. coli* strain, the 1306 bp fragment containing both left and right ITRs of ChAd6 as well as pIX gene fragment was excised by PmeI digestion from pNEBChAd6-RLIdE1 and transferred to a different plasmid vector obtained by PCR amplification with the olinucleotides 5'-GATCTAGTTAGTTTAAACGAATTCGGATCTGCGACGCG-3' (SEQ ID NO: 33) and 5' TTCGATCATGTTTAAACGAAATTAAGAATTCGGATCC-3' (SEQ ID NO: 34) from pMRKAd5SEAP. This final ligation step generated the ChAd6 shuttle vector pARSChAd6-3.

Construction of a Subgroup C Shuttle Vector

The ChAd3 viral genome was fully sequenced (SEQ ID NO: 1) and the information obtained was used to construct a shuttle vector to facilitate cloning by homologous recombination of subgroup C chimpanzee adenovirus.

Briefly, the shuttle vector used to clone subgroup C chimp adenovirus, referred to herein as pChAd3EGFP was constructed as follows: a ChAd3 DNA fragment (nt 3542-4105) containing pIX coding region was amplified by PCR with the oligonucleotides 5'-TATTCTGCGATCGCTGAGGTGGGTGAGTGGGCG-3' (SEQ ID NO: 35) and 5'-TAGGCGCGCCCTTAAACGGCATTTGTGGGAG-3' (SEQ ID NO: 36) digested with SgfI- AscI then cloned into pARSCV32-3 digested with SgfI- AscI, generating pARS-ChAd3D. ChAd3 right end (nt 37320-37441) was amplified by PCR with oligonucleotides 5'-CGTCTAGAAGACCCGAGTCTTACCAGT-3' (SEQ ID NO: 37) and 5'-CGGGATCCGTTTAAACCATCATCAATAATATACCTTATT-3' (SEQ ID NO: 38) digested with XbaI and BamHI then ligated to pARS-ChAd3D restricted with XbaI and BamHI, generating pARS-ChAd3RD. ChAd3 viral DNA left end (nt 1-460) was amplified by PCR with oligonucleotides 5'-ATGGAATTCGTTTAAACCATCATCAATAATATACCTT-3' (SEQ ID NO: 39) and 5'-ATGACGCGATCGCTGATATCCTATAATAATAAAACGCAGACTTTG-3', (SEQ ID NO: 40) digested with EcoRI and SgfI then cloned pARS-ChAd3RD digested with EcoRI and SgfI, thus generating pARS-ChAd3RLD. The viral DNA cassette was also designed to contain restriction enzyme sites (PmeI) located at the end of both ITR's so that digestion will release viral DNA from plasmid DNA.

Construction of a Subgroup B Shuttle Vector

The construction of subgroup B shuttle followed the already described strategy for subgroup C and D/E shuttle constructions. In brief, pARS-ChAd3RLD was modified by substituting the left end, the pIX region, the right end with the corresponding fragments of ChAd30. In addition the E4 region of ChAd30 was substituted with Ad5 E4orf6 that was cloned under the ChAd30 E4 promoter control. The shuttle plasmid was denominated pChAd30 EGFP shuttle vector.

Construction of ΔE1 Chimp Adenoviral Vectors

Subgroup B: Subgroup B chimp adenovirus vectors were constructed by homologous recombination in *E. coli* strain BJ5183. BJ5183 cells were co-transformed with pChAd30EGFP shuttle vector digested with BstEII and Bst1107I and ChAd8 and ChAd30, purified viral DNA. Homologous recombination between pIX genes, right ITR DNA sequences present at the ends of linearized pChAd30EGFP shuttle and viral genomic DNA allowed its insertion in the plasmid vector, deleting at the same time the E1 region that was substituted by EGFP expression cassette. Expression cassettes based on

human cytomegalovirus (HCMV) promoter and bovine growth hormone polyadenylation signal (Bgh polyA) were constructed to express secreted alkaline phosphatase (SEAP), EGFP, HIV gag, HCV NS region (as described in fig.3 for ChAd6 shuttle vectors) as well as tumor-associated antigens like CEA and HER2/neu from human and Rhesus monkey origin. All expression cassette were inserted into ChAd30 vectors by homologous recombination.

Subgroup C: Subgroup C chimp adenovirus vectors were constructed by homologous recombination in *E. coli* strain BJ5183. BJ5183 cells were co-transformed with pChAd3EGFP shuttle vector digested with BstEII and Bst1107I and ChAd3, ChAd11, ChAd19 and ChAd20 purified viral DNA. Homologous recombination between pIX genes, right ITR DNA sequences present at the ends of linearized pChAd3EGFP and viral genomic DNA allowed its insertion in the plasmid vector, deleting at the same time the E1 region that was substituted by EGFP expression cassette. Expression cassettes based on human cytomegalovirus (HCMV) promoter and bovine growth hormone polyadenylation signal (Bgh polyA) were constructed to express secreted alkaline phosphatase (SEAP), EGFP, HIV gag, HCV NS region (as described in fig.3 for ChAd6 shuttle vectors) as well as tumor-associated antigens like CEA and HER2/neu from human and Rhesus monkey origin.

Subgroups D and E: In order to construct ΔE1 vectors based on subgroup D and E chimp adenovirus, the shuttle vector pARS ChAd6-3 was digested with AscI and co-transformed into *E. coli* strain BJ5183 with CV32, CV33, CV68, ChAd4, ChAd5, ChAd6, ChAd7, ChAd9, ChAd10 and ChAd16 purified viral DNA. Homologous recombination between DNA sequences from pIX genes and right ITR present at the ends of linearized pARS ChAd6-3 and viral genomic DNA allowed its insertion in the plasmid vector, deleting at the same time the E1 region (Figures 2 and 4).
Expression cassettes based on human cytomegalovirus (HCMV) promoter and bovine growth hormone poly-adenylation signal (Bgh polyA) were constructed to express secreted alkaline phosphatase (SEAP), EGFP, HIV gag, HCV NS genes (Figure 3) as well as tumor-associated antigens like CEA and HER2/neu of human and Rhesus monkey origin. All the expression cassette were inserted into the single SnaBI site of pARS ChAd6-3 vector to be transferred by homologous recombination into the ΔE1 adenovirus pre-plasmids as described in Figure 4.

Rescue and Amplification of ΔE1 Vectors

$5X10^6$ PER.C6™ cells planted on 6cm cell culture dishes were transfected with 10 micrograms of cloned viral vector released from plasmid sequences by endonuclease digestion. DNA transfection was performed using Lipofectamine (Invitrogen). Transfected cells and culture medium were collected 5-10 days post-transfection and lysed by freeze-thaw. Rescued vectors were then amplified by serial passaging on 293 or PER.C6™ cells. A large-scale amplification was performed by infecting cells planted on 5-10 cell-factories (NUNC, Inc.) on a total of $1-2x10^9$ cells. A purified vector preparation was

obtained on cesium chloride gradient by two ultra-centrifuge runs, dialyzed against PBS containing 10% glycerol and stored at –70°C in aliquots.

Example 3: Neutralization Studies

Neutralization assays were carried out in order to evaluate the prevalence in human sera of neutralizing antibodies against the chimpanzee adenoviruses disclosed herein. The assay evaluated the effects of serum preincubation on the ability of chimp adenoviruses carrying the gene for secreted alkaline phosphatase (SEAP) to transduce human 293 cells. The neutralization titer is defined as the dilution of serum giving a 50% reduction of the SEAP activity observed in the positive control with the virus alone.

From $2x10^6$ to $1.5x10^7$ physical particles of CV33-SEAP, CV32-SEAP and ChAd3-SEAP vector were diluted in 100 µl of complete medium and added to an equal volume of human or chimp serum diluted in complete medium. Each serum samples was tested at various dilutions (five 4-fold increments starting from 1/18 dilution through 1:4608). Samples were pre-incubated for one hour at 37°C and then added to 293 cells seeded into 96-well plates ($3x10^4$ cells/well). The inoculum was removed after one hour of incubation, the cells were re-fed with fresh medium and, 24 hours later, 50 µl of medium was removed and the SEAP activity was measured by a chemiluminescent assay. The neutralization titer is defined as the dilution of serum giving a 50% reduction of the SEAP activity observed in the positive control with the virus alone. A panel of 100 human sera was tested for ChAd neutralization activity. In parallel the same panel was tested on Ad5 SEAP vector.

Table 2. Prevalence of Neutralizing Antibodies Against Chimpanzee Adenovirus

	Virus						
titer	**hAd5**	**CV32**	**CV33**	**ChAd3**	**ChAd30**	**ChAd9**	**ChAd10**
< 200	**77%**	**96%**	**100%**	**92%**	**100%**	**92%**	**100%**
> 200	**33%**	**4%**	**0%**	**8%**	**0%**	**6%**	**0%**

The result provided in Table 2 indicates that a very low prevalence in human sera of neutralizing antibodies directed against vector derived from chimpanzee adenoviruses. Only four sera showed a titer over the threshold of 200 on CV32 vector while 8 showed a titer over 200 on ChAd3 SEAP vector. On the contrary, the panel of chimp sera examined showed a very high prevalence of anti-Chimp Ad immunity. These findings confirm that as expected, vectors based on chimp Ads have a very little chance to be neutralized in humans. Therefore they represents an ideal solution to the problem of the pre-

existing anti-human Ad immunity that limits the administration of viral vectors based on common human Ad serotypes such as Ad5.

Example 4: ChAd Vector Tropism

Gene transfer efficacy mediated by Ad5 and ChAd vectors was assessed by EGFP expression on a panel of human primary cells of different histological origin. Human chondrocytes, osteoblasts, keratinocytes, melanocytes, skeletal muscle cells and melanocytes were cultivated according to manufacturer indication. Human monocytes, immature and mature dendritic cells (DC) were obtained as described (Romani, N. et al. 1996, *J. Immunol. Methods*, 196,137.). Transduced, fluorescent cells were detected by FACS analysis. The panel of human primary cells tested includes cells that are important target cells for different therapeutic strategies based on *in vivo* as well as *ex vivo* gene transfer in the field of cardiovascular disease, rheumatoid arthritis, tissue engineering (bone, skin, and cartilage), and vaccination. The results presented in Figure 38A-D suggests that different chimp adenoviruses can recognize receptors alternative to CAR as demonstrated by the differential efficiency of infection of the different cell types.

MURINE IMMUNIZATION STUDIES

METHODS AND MATERIALS

Immunization Protocols and Splenocyte /PBMC Preparation

Immunizations: Mice were immunized with the selected adenoviruses diluted in 0.1 ml of buffer. Each vector dose was divided in two aliquot of 50 µl and injected in both quadriceps of mice.

Splenocyte Preparation: Mice were sacrificed 3 weeks post-injection and their spleens excised and transferred in 10 ml of R10 (10% FCS, 55mM 2-mercaptoethanol, 1M HEPES buffer, 2mM L-glutamine, 1X penicillin-streptomicine solution in RPMI medium 1640). Spleens were minced through a steel screen and, after the screen was washed with 2 ml of R10, splenocytes were transferred in a 50 ml Falcon tube and centrifuged at 1200 rpm, 10 min, room temperature (rt). Supernatant was removed and 3 ml of ACK lysis buffer (Gibco BRL Formulation#79-0422DG) were added. Cells were incubated 5 min, rt. 45 ml of 1X PBS were added and tubes were centrifuged as above. After washing with 30 ml of R10, cells were resuspended in 5 ml of R10, filtered through a 70 m Nylon cell strainer (Falcon 2350). 10µl of cells were diluted with 990 µl Turk's solution (Merck 040417345) and counted. Cells were finally diluted to 10^7 cells /ml in R10.

Peripheral blood mononuclear cell (PBMC) preparation: Mice blood samples (150 ul) were transferred to 2ml eppendorf tubes with 50ul PBS/2% EDTA. 1 ml ACK buffer was added to each tube. Gently mixed and incubated at RT for 5 min. Samples were centrifuged at 1500rpm in microcentrifuge

for 5 min. Supernatant was discharged white cell pellets deriving from the same immunized cohorts were combined. ACK buffer incubation was repeated then pellets of PBMC were resuspended in 1 ml of R10 medium.

IFN-γ ELISPOT Assay

Millipore MAIP 45 plates were coated with 100 µl/well of purified rat anti-mouse IFN-γ monoclonal antibody (Pharmingen, cat. 551216) diluted at 2.5 µg/ml in PBS and incubated over-night (o/n) at 4^0C. Plates were washed 2X with sterile PBS and un-specific binding sites were blocked by incubation for 2hrs in the CO_2 incubator with 200 µl/well of R10. In the immunization experiments with Ad vectors expressing HIV gag, a 9-mer peptide (AMQMLKETI, a CD8 HIV gag epitope mapped in Balb/C mice) (SEQ ID NO: 47) was diluted to 2 µg/ml in R10 and added to the wells in the amount of 50 µl/well. In immunization experiments conducted with HCV-NS expressing vectors, a pool of peptides covering NS3 helicase domain as well a 9-mer peptide representing a mapped CD8 epitope comprised in helicase domain were used. Immunization experiments with ChAds expressing human CEA antigen were evaluated by pools of overlapping 15-mer peptides covering the entire amino acid sequence. As controls DMSO and Concanavalin A were used. Cells were added to each well at the amount of $5X10^5$ and $2.5X10^5$. After an o/n incubation in the CO_2 incubator, plates were washed with 0.05% Tween 20/PBS and 50 µl/ well of biotinylated rat anti-mouse IFN-γ monoclonal antibody (PharMingen cat. 554410) diluted 1/250 in assay buffer (5%FBS, 0.005% Tween20, PBS) were added. Plates were incubated o/n at 4^0C and washed as above. Streptavidin-alkaline phosphatase conjugate (BD554065) was diluted 1 /2500 in assay buffer and added in the amount of 50 µl/well for 2 hrs rt. After washing, plates were developed adding 50 µl/well of BCIP/NBT1-step solution (Pierce 34042). Reaction was stopped by washing wells with deionized water. Spots were automatically counted by an ELISPOT reader.

Murine IFN-γ Intracellular Staining (ICS)

Splenocytes were diluted at $2X10^6$ cells in 1 ml of R10 and stimulated with the same antigens described above at the concentration of 2 µg/ml. As controls, DMSO and Staphylococcal Enterotoxin B (SEB) were used. After an overnight incubation in the CO_2 incubator, cells were washed with FACS buffer (1% FCS, 0.01% NaN3, PBS) and purified anti-mouse CD16/CD32 Fc block (clone 2.4G2, Pharmingen cat. 553142) was diluted 1/25, added in the amount of 100 µl/sample and incubated for 15min at 4^0C. Cells were washed in FACS buffer and APC conjugated anti-mouse CD3e (clone 145-2C11, Pharmingen #553066), PE conjugated anti-mouse CD4 (clone L3T4, BD Pharmingen cat. 553142) and PerCP conjugated anti-mouse CD8a (clone 53-6.7, Pharmingen cat. 553036) diluted 1:50 in FACS buffer were added in the amount of 100 µl/sample. Cells were incubated 30 min rt, washed, fixed and permeabilized (Becton Dickinson, FACS Perm 2) and incubated with FITC conjugated anti-mouse IFN-γ

Pharmingen cat.554411) diluted 1:50 in PermWash (100 ul/sample) for 30 min at RT. After washing cells were resuspended in 500 ul 1% formaldehyde/PBS and intracellular cytokine staining (ICS) analyzed on a FACS-Calibur flow cytometer, using CellQuest software (Becton Dickinson).

Example 5: ChAd Vectors Elicit Strong CMI Responses in Mice

The ability of the ChAd vectors disclosed herein to elicit a cell-mediated immune response (CMI) was evaluated in mice using vectors expressing an HIV gag transgene. Briefly, groups of 5 Balb/C mice were injected with ten-fold increasing doses of the different vectors starting from 10^5 up to 10^{10} vp/mouse.

The strength of the immune response was determined three weeks after the injection by quantifying gag-specific CD8+ T cells in the splenocytes. The number of IFN-γ secreting CD8+ T cells was determined by ELISPOT assay or by IFN-γ intracellular staining and FACS analysis after stimulation *in vitro* with a peptide reproducing a gag CD8+ T cell epitope mapped in Balb/C mice.

The results obtained from the 5 immunized animals, reported in Table 3, are expressed as spot forming cells per 10^6 splenocytes. Shown are the number of spot forming cells per million splenocytes following incubation with 9-mer CD8+ gag epitope or with gag peptide pool. The gag peptide pool consisted of 20-aa peptide overlapping by 10aa encompassing the entire gag sequence. Positive values are reported in bold.

The data provided in Table 3 indicate that the administration of the ChAd vectors disclosed and claimed herein elicits a strong cell mediated immune response which is comparable to the response elicited by hAd5. By looking at the lowest vector dose resulting in a positive immunization result (immunization breakpoint), we ranked the potency of the different vectors being subgroup C ChAd3gag the most potent with a breakpoint at 10^6 pp vector dose. Ranking by immunization break-points is shown in Figure 33.

Table 3. Gag-Specific T Cell Response in Balbc Mice Immunized with Chimpanzee Ad Vectors

Vaccination	10^5 vp		10^6 vp		10^7 vp		10^8 vp		10^9 vp		10^10 vp	
	mock	Gag	mock	Gag	mock	Gag	mock	Gag	mock	Gag	mock	Gag
ChAd3DE1gag	1	neg	1	944	1	1298	1	1258	NT	NT	NT	NT
	3	neg	1	1039	1	1958	1	1962	NT	NT	NT	NT
	1	neg	1	859	1	1923	1	1931	NT	NT	NT	NT
	1	neg	1	1620	1	1386	1	1369	NT	NT	NT	NT
	1	neg	1	1529	5	1442	4	1436	NT	NT	NT	NT
CV33DE1gag	NT	NT	1	neg	2	475	1	2910	NT	NT	NT	NT
	NT	NT	1	neg	1	433	1	401	NT	NT	NT	NT
	NT	NT	1	neg	1	243	1	634	NT	NT	NT	NT
	NT	NT	1	neg	1	505	2	3457	NT	NT	NT	NT
	NT	NT	1	neg	1	683	2	1684	NT	NT	NT	NT
CV68DE1gag	NT	NT	3	neg	1	340	2	332	0	406	2	635
	NT	NT	1	neg	1	512	0	536	1	256	3	1172
	NT	NT	0	neg	2	458	3	944	2	462	2	505
	NT	NT	7	neg	0	148	1	519	0	488	2	1184
	NT	NT	0	neg	2	1418	1	243	0	240	1	789
ChAd9DE1gag	NT	NT	1	neg	7	369	1	609	NT	NT	NT	NT
	NT	NT	1	neg	1	508	1	739	NT	NT	NT	NT
	NT	NT	1	neg	1	299	16	291	NT	NT	NT	NT
	NT	NT	1	neg	2	507	8	926	NT	NT	NT	NT
	NT	NT	0.5	neg	1	36	40	1034	NT	NT	NT	NT
ChAd10DE1gag	NT	NT	1	neg	1	83	1	822.5	NT	NT	NT	NT
	NT	NT	1	neg	1	42.5	1	1033	NT	NT	NT	NT
	NT	NT	1	neg	1	48	1	1339.5	NT	NT	NT	NT
	NT	NT	1	neg	4	51	1	1132	NT	NT	NT	NT
	NT	NT	1	neg	1	466.5	1	521.5	NT	NT	NT	NT
ChAd6DE1gag	NT	NT	1	neg	1	34	1	721	NT	NT	NT	NT
	NT	NT	1	neg	10	4	1	560	NT	NT	NT	NT
	NT	NT	1	neg	1	24	1	624	NT	NT	NT	NT
	NT	NT	1	neg	1	225	3	3002	NT	NT	NT	NT
	NT	NT	1	neg	1	276	4	1738	NT	NT	NT	NT
ChAd11DE1gag	1	neg	1	neg	0	573	NT	NT	NT	NT	NT	NT
	0	neg	0	neg	0	919	NT	NT	NT	NT	NT	NT
	0	neg	1	neg	1	1438	NT	NT	NT	NT	NT	NT
	2	neg	0	neg	0	0	NT	NT	NT	NT	NT	NT
	1	neg	1	neg	0	456	NT	NT	NT	NT	NT	NT
ChAd20DE1gag	0	neg	0	neg	0	1	NT	NT	NT	NT	NT	NT
	2	neg	0	neg	0	408	NT	NT	NT	NT	NT	NT
	0	neg	0	neg	0	414	NT	NT	NT	NT	NT	NT
	1	neg	0	neg	0	2	NT	NT	NT	NT	NT	NT
	0	neg	0	neg	1	311	NT	NT	NT	NT	NT	NT
ChAd7DE1gag	NT	NT	1	neg	1	neg	1	1044	NT	NT	NT	NT
	NT	NT	3	neg	1	neg	1	606	NT	NT	NT	NT
	NT	NT	1	neg	8	neg	1	407	NT	NT	NT	NT
	NT	NT	1	neg	1	neg	2	567	NT	NT	NT	NT
	NT	NT	1	neg	3	neg	1	1677	NT	NT	NT	NT
CV32DE1gag	NT	NT	NT	NT	1	neg	0	83	0	291	0	194
	NT	NT	NT	NT	3	neg	0	382	0	805	2	380
	NT	NT	NT	NT	0	neg	1	97	0	136	1	501
	NT	NT	NT	NT	1	neg	5	96	4	1162	0	1115
	NT	NT	NT	NT	2	neg	1	328	NT	NT	0	596
ChAd4DE1gag	NT	NT	1	neg	0	neg	0	0	NT	NT	NT	NT
	NT	NT	0	neg	0	neg	0	159	NT	NT	NT	NT
	NT	NT	0	neg	0	neg	0	1	NT	NT	NT	NT
	NT	NT	1	neg	0	neg	0	234	NT	NT	NT	NT
	NT	NT	1	neg	0	neg	1	0	NT	NT	NT	NT
ChAd16DE1gag	NT	NT	0	neg	0	neg	0	243	NT	NT	NT	NT
	NT	NT	0	neg	0	neg	1	296	NT	NT	NT	NT
	NT	NT	0	neg	2	neg	1	68	NT	NT	NT	NT
	NT	NT	0	neg	0	neg	1	433	NT	NT	NT	NT
	NT	NT	1	neg	0	neg	1	28	NT	NT	NT	NT

5

Example 6: ChAd3 and CV33 GAG Vectors Elicit a CMI Response Characterized by GAG-Specific CD8+ T Cells

In order to characterize the CMI response elicited in response to the ChAd vectors comprising HIV gag transgene, splenocytes pooled from cohorts of five mice immunized with different doses of vector were analyzed by intracellular IFN-γ staining. The data shown in table 3 and table 4 were collected in separate experiments.

Splenocytes were diluted at $2X10^6$ cells in 1 ml of R10 and stimulated with the same antigens described above at the concentration of 2 μg/ml. As controls, DMSO and SEB (Staphylococcal Enterotoxin B) were used. After an o/n incubation in the CO_2 incubator, cells were washed with FACS buffer (1% FCS, 0.01% NaN3, PBS) and purified anti-mouse CD16/CD32 Fc block (clone 2.4G2, Pharmingen cat. 553142) was diluted 1/25, added in the amount of 100 μl/sample and incubated for 15min at 4^0C. Cells were washed in FACS buffer and APC conjugated anti-mouse CD3e (clone 145-2C11, Pharmingen #553066), PE conjugated anti-mouse CD4 (clone L3T4, BD Pharmingen cat. 553142) and PerCP conjugated anti-mouse CD8a (clone 53-6.7, Pharmingen cat. 553036) diluted 1:50 in FACS buffer were added in the amount of 100 μl/sample. Cells were incubated 30 min rt, washed, fixed and permeabilized (Becton Dickinson, FACS Perm 2) and incubated with FITC conjugated anti-mouse IFN-γ Pharmingen cat.554411) diluted 1:50 in PermWash (100 ul/sample) for 30 min at RT. After washing cells were resuspended in 500 ul 1% formaldehyde/PBS and analyzed on a FACS-Calibur flow cytometer, using CellQuest software (Becton Dickinson).

Table 4 provides data summarizing the percentage of gag-specific CD3+T cells that were either gag-specific CD8+ or CD4+ T cells. Positive results are reported in bold. The data provided herein indicate that the cellular profile of the immune response elicited by ChAd vectors derived from viruses classified into different serotype subgroups (i.e., subgroups C, D and E) are similar and all of the gag-specific responses characterized predominantly by CD8+ T cells. In addition, it is noted that at high vector doses a gag-specific CD4+ response becomes evident in all immunization experiments. The ICS assay confirmed that ChAd3 vector can stimulate anti-gag CD8+ response at 10^6 vector dose.

Table 4. Characterization of Gag-Specific T Cells in Mice Immunized with Chimp Adenovirus Vectors of Different Subgroups

vaccine		10^5		10^6		10^7		10^8		10^9	
		DMSO	gag	DMSO	gag	DMSO	gag	DMSO	gag	DMSO	gag
ChAd3DE1gag	%CD8+CD3+	NT	NT	0.01%	**4.65%**	0.01%	**17.15%**	0.04%	**24.71%**	NT	NT
	%CD4+CD3+	NT	NT	0.00%	0.07%	0.03%	0.08%	0.04%	**0.28%**	NT	NT
CV33DE1gag	%CD8+CD3+	NT	NT	0.02%	0.01%	0.01%	**0.83%**	0.03%	**8.69%**	NT	NT
	%CD4+CD3+	NT	NT	0.00%	0.00%	0.00%	0.04%	0.01%	**0.10%**	NT	NT
ChAd9DE1gag	%CD8+CD3+	NT	NT	0.02%	0.01%	0.01%	**0.68%**	NT	NT	0.04%	**4.73%**
	%CD4+CD3+	NT	NT	0.00%	0.00%	0.00%	0.00%	NT	NT	0.00%	0.01%
ChAd10DE1gag	%CD8+CD3+	NT	NT	0.02%	0.01%	0.01%	**0.57%**	NT	NT	0.02%	**5.04%**
	%CD4+CD3+	NT	NT	0.00%	0.00%	0.00%	0.00%	NT	NT	0.00%	0.01%
ChAd6DE1gag	%CD8+CD3+	NT	NT	0.00%	0.01%	0.00%	**0.59%**	0.01%	**14.28%**	NT	NT
	%CD4+CD3+	NT	NT	0.00%	0.00%	0.00%	0.05%	0.01%	**0.12%**	NT	NT
ChAd7DE1gag	%CD8+CD3+	NT	NT	0.01%	0.02%	0.01%	0.00%	0.02%	**5.00%**	NT	NT
	%CD4+CD3+	NT	NT	0.00%	0.01%	0.00%	0.00%	0.01%	**0.21%**	NT	NT

Example 7: ChAd Vectors Elicit HCV NS-Specific T Cell Response

The potency of CV32-NSmut and CV33-NSmut vectors was evaluated in C57/Black6 mice relative to the potency of MRKAd6NSmut. The animals were injected with 10-fold increasing doses of vector starting from 10^7 up to 10^9 vp/mouse. CMI was analyzed 3 weeks after a single injection by IFN-γ ELISPOT and IFN-γ intracellular staining by stimulating T cells with a 9-mer peptide reproducing a CD8+ T cell epitope mapped in the helicase domain of NS3 protein. The data provided in Table 5 summarize the number of spot-forming cells per million splenocytes following incubation in absence (mock) or in presence of NS3 9-mer peptide.

The data indicate that both CV32 and CV33 vectors expressing HCV-NS stimulate strong T cell responses. Based on the observation that the first positive result for the CV32 vector was obtained by injecting 10^9 vp/dose, the immunization potency of CV32DE1E3 NSmut vector appears to be approximately 100-fold lower than human subgroup C Ad6DE1E3 NSmut vector. The parallel experiment with MRKAd6NSmut indicated that a dose of 10^7 vp/animal was sufficient to stimulate cell mediated immunity. Therefore, these results confirm the lower immunization potency of CV32-derived vectors relative to human subgroup C vectors (such as hAd5 and hAd6) that was also observed in the experiment with gag expressing vectors (see Table 3).

Table 5. HCV NS-Specific T Cell Response in Mice Immunized with Mrkad6 Nsmut, CV32NSmut or CV33NSmut

Vaccination	10^7 vp		10^8 vp		10^9 vp		10^10 vp	
	Mock	NS3	mock	NS3	mock	NS3	mock	NS3
MRKAd6NSmut	1	345	1	449	NT	NT	NT	NT
	1	248	1	1590	NT	NT	NT	NT
	1	1	1	549	NT	NT	NT	NT
	1	262			NT	NT	NT	NT
					NT	NT	NT	NT
CV33NSmut	1	1	1	195	2	338	NT	NT
	1	2	1	409	1	1136	NT	NT
	1	1	1	396	1	497	NT	NT
	1	2	2	172	1	344	NT	NT
	1	237			1	163	NT	NT
CV32NSmut	neg	neg	1	181	1	118	1	176
	neg	neg	1	71	1	239	1	238
	neg	neg	1	56	1	862	1	555
	neg	neg	1	459	1	219	1	545
	neg	neg	1	195	1	123	1	578

Example 8: Anti -Ad5 Pre-Existing Immunity Does Not Abrogate Anti-GAG CMI Elicited by ChAd3gag

To evaluate the impact on ChAd3 immunization of the pre-existing immunity against the high seroprevalent Ad5, 4 cohorts of 5 BalbC mice were pre-immunized with two injection of 10^{10} vp of Ad5 wt in the quadriceps at week 0 and 2. As control, 2 cohorts of 5 mice were injected at the same time points with buffer only. Cohorts of Ad5 pre-immunized mice were then immunized with 10^6 and 10^7 vp/mouse of either Ad5gag or ChAd3gag vectors. Cohorts of control (naïve) mice were immunized with 106 vp/mouse of Ad5gag or ChAd3gag vectors.

Anti-Ad5 and ChAd3 neutralizing immunity was evaluated at week 4 by the neutralization assay described above using Ad5 and ChAd3 SEAP vectors. Anti-gag immunity was evaluated by ELISPOT analysis on purified splenocytes stimulated with gag 9-mer peptide containing a gag epitope mapped in BalbC mice. The results reported in figure 36 demonstrated that Anti-Ad5 immunity does not abrogate anti-gag CMI elicited by ChAd3gag while, as expected, anti-Ad5 immunity completely block Ad5gag immunization.

Example 9: ChAd3hCEA Immunization Elicits a Strong CEA-Specific Immune Response in Transgenic Mice Expressing Human CEA

The ability of the ChAd vectors disclosed and claimed herein to elicit an immune response against a self-antigen therefore breaking the tolerance was also evaluated in transgenic mice expressing human CEA (Clarke, P *et al. Cancer Res.* (1998) 58(7):1469-77.)

Cohorts of 8 mice were injected in the quadriceps with 10^10 vp of ChAd3hCEA or Ad5hCEA as already described. The immune response against CEA was followed weekly up to day 75 on PBMC stimulated with a pool of 15-mer peptides encompassing human CEA amino acid sequence from aa 497 to the end (aa 703). Anti-CEA immunity was evaluated by ICS determining CD4-CD8+ T cells secreting interferon-γ in response to CEA peptide pool incubation.

The results reported in figure 37 demonstrate that ChAd3hCEA vector immunization stimulate a more sustained CD8+ T cell response against human CEA than Ad5 expressing the same transgene.

PRIMATE IMMUNIZATION STUDIES

METHODS AND MATERIALS

Immunization Protocol

The ability of the ChAd vectors disclosed and claimed herein to elicit CMI in Rhesus macaques (referred to herein as monkeys) was also evaluated. The macaques were anesthetized (ketamine/xylazine) and the vaccines were delivered i.m. in 0.5-mL aliquots into both deltoid muscles using tuberculin syringes (Becton-Dickinson). In all cases the macaques were between 3-10 kg in weight, and the total dose of each vaccine was administered in 1 mL of buffer.

Sera and peripheral blood mononuclear cells (PBMC) were prepared from blood samples collected at several time points during the immunization regimen. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council.

ELISPOT Assay

The IFN-γ ELISPOT assays for rhesus macaques were conducted following a previously described protocol (Allen *et al.*, 2001 *J. Virol.* 75(2):738-749), with some modifications. For gag-specific stimulation, a peptide pool was prepared from 20-aa peptides that encompass the entire HIV-1 gag sequence with 10-aa overlaps (Synpep Corp., Dublin, CA). For HCV NS-specific stimulation 6 peptide pools were prepared from 15-aa peptides that encompass the entire HCV-NS sequence from NS3 to NS5b with 10-aa overlaps.

HER2/neu and CEA-specific stimulations were performed with 15-aa peptides that encompass the entire protein sequence with 10-aa overlaps.

To each well, 50 µL of 2-4 x 10^5 peripheral blood mononuclear cells (PBMCs) were added; the cells were counted using Beckman Coulter Z2 particle analyzer with a lower size cut-off set at 80 fL. Either 50 µL of media or the gag peptide pool at 8 µg/mL concentration per peptide was added to the PBMC. The samples were incubated at 37°C, 5% CO_2 for 20-24 hrs. Spots were developed accordingly and the plates were processed using custom-built imager and automatic counting subroutine based on the ImagePro platform (Silver Spring, MD); the counts were normalized to 10^6 cell input.

Intracellular Cytokine Staining (ICS)

To 1 ml of 2 x 10^6 PBMC/mL in complete RPMI media (in 17x100mm round bottom polypropylene tubes (Sarstedt, Newton, NC)), anti-hCD28 (clone L293, Becton-Dickinson) and anti-hCD49d (clone L25, Becton-Dickinson) monoclonal antibodies were added to a final concentration of 1 µg/mL. For gag-specific stimulation, 10 µL of the peptide pool (at 0.4 mg/mL per peptide) were added. Similar conditions were used for HCV NS-specific stimulation. The tubes were incubated at 37 °C for 1 hr., after which 20 µL of 5 mg/mL of brefeldin A (Sigma) were added. The cells were incubated for 16 hr at 37 °C, 5% CO_2, 90% humidity. 4 mL cold PBS/2%FBS were added to each tube and the cells were pelleted for 10 min at 1200 rpm. The cells were re-suspended in PBS/2%FBS and stained (30 min, 4 °C) for surface markers using several fluorescent-tagged mAbs: 20 µL per tube anti-hCD3-APC, clone FN-18 (Biosource); 20 µL anti-hCD8-PerCP, clone SK1 (Becton Dickinson, Franklin Lakes, NJ); and 20 µL anti-hCD4-PE, clone SK3 (Becton Dickinson). Sample handling from this stage was conducted in the dark. The cells were washed and incubated in 750 µL 1xFACS Perm buffer (Becton Dickinson) for 10 min at room temperature. The cells were pelleted and re-suspended in PBS/2%FBS and 0.1 µg of FITC-anti-hIFN-γ, clone MD-1 (Biosource) was added. After 30 min incubation, the cells were washed and re-suspended in PBS. Samples were analyzed using all four color channels of the Becton Dickinson FACSCalibur instrument. To analyze the data, the low side- and forward-scatter lymphocyte population was initially gated; a common fluorescence cut-off for cytokine-positive events was used for both $CD4^+$ and $CD8^+$ populations, and for both mock and gag-peptide reaction tubes of a sample.

Example 10: A Homologous Prime-Boost Regimen Using ChAd ΔE1-gag Vectors Elicits Gag-Specific T Cells in Monkeys

Cohorts of 3 animals were given intramuscular injection at week 0 and week 4 of either of the following constructs: 10^10 vp of CV-32ΔE1-gag; or 10^10 vp CV33ΔE1-gag; or 10^10 vp and 10^8 vp MRKAd5ΔE1gag. PBMCs collected at regular 4-wks intervals were analyzed in an ELISPOT assay. The results provided in Table 6, which indicate the number of spot-forming cells per million PBMC

following incubation in absence (mock) or presence of Gag peptide pool establish that both CV32ΔE1-gag and CV-33ΔE1gag are able to induce significant levels of gag-specific T cells in non-human primates. It is interesting to note that after a single dose (wk 4), the CV32ΔE1-gag responses were comparable to MRKAd5 ΔE1-gag 10^8 vp dose and lower than that of MRKAd5-gag 10^10 vp/dose. CV33ΔE1-gag 10^10 vp/dose induces a response comparable to that of MRKAd5-gag 10^10 vp/dose. This result was confirmed at week 8 after the second dose.

Table 6. Gag-Specific T Cell Response in Monkeys Immunized with Mrkad5 ΔE1-Gag, CV32ΔE1-Gag, CV33ΔE1-Gag

Vaccination T=0	vector dose	Monk #	Pre-bleed		T=4		T=8	
			Mock	Gag	Mock	Gag	Mock	Gag
CV32ΔE1gag	10^10 vp	01C023	1	0	14	353	3	278
		01C029	1	3	13	605	3	419
		01C032	1	0	5	274	1	179
CV33ΔE1gag	10^10 vp	01C033	0	0	9	1545	1	659
		01C036	4	5	4	1540	13	881
		01D303	0	3	19	949	10	628
MRKAd5gag	10^8 vp	01D267	0	0	4	473	0	341
		01D279	1	4	44	831	6	336
		01D284	4	5	4	264	5	129
MRKAd5gag	10^10 vp	99C218	0	3	5	2500	0	1580
		99C227	6	1	4	529	5	365
		99D185	ND	ND	0	425	0	310

Example 11: ChAd Vectors Elicit a HCV NS-Specific T-Cell Response in a Heterologous Prime-Boost Regimen

In a separate experiment, groups of two and three monkeys were given immunization at week 0, 4 of MRK Ad6NSoptmut vector at 10^8 or 10^10 vp per animal. The animals were boosted with the same virus at the same dose at week 24 and then boosted again at week 104 with CV33-NSmut at 10^10 vp per animal. The results are presented in Tables 7 and 8 which summarize the number of spot-forming cells per million PBMC following incubation in absence (mock) or presence of HCV NS peptide pool.

T cell immunity, as assessed by IFN-γ ELISPOT, showed a peak response at week 4 after the first dose in the animals injected with 10^10 vp (Table 8) and at week 8 (post-dose 2) in the animals injected at 10^8 (Table 7). The response was not boosted by the injection at week 24 ("homologous boost"), while a strong boost effect was observed after the injection with CV33-NSmut ("heterologous boost").

Table 7. HCV NS-Specific T Cell Response in Monkeys Immunized with MRK Ad6NSoptmut At 10^8 Vp/Animal and Boosted with CV33-Nsmut

Vaccine	MRKAd6NSoptmut 10^8 vp								CV33-NSmut 10^10 vp			
time point	post-priming I dose T=4		post-priming II dose T=8		pre-homologous boost T=24		post-homologous boost T=28		pre-heterologous boost T=104		post-heterologous boost T=108	
monkey	*95116*	*138T*	*95116*	*138T*	*95116*	*138T*	*95116*	*138T*	*95116*	*138T*	*95116*	*138T*
poolF	44	112	77	124	115	176	105	55	120	150	188	2228
poolG	20	2110	86	1975	201	1105	94	884	120	192	96	4590
poolH	12	18	54	22	169	221	28	9	81	33	447	543
pooll	14	53	62	47	163	189	96	18	80	67	71	515
poolL	33	86	58	44	353	608	235	33	110	131	224	308
poolM	184	75	168	138	204	336	67	44	55	46	2028	1570
DMSO	14	3	44	7	104	79	33	6	57	40	33	65

Table 8. HCV NS-Specific T Cell Response In Monkeys Immunized MRK Ad6NSoptmut At And 10^10 vp/Animal And Boosted With CV33-Nsmut

Vaccine	MRKAd6NSoptmut 10^10 vp												CV33-NSmut 10^10					
time point	post-priming I dose T=4			post-priming II dose T=8			pre-homologous boost T=24			post-homologous boost T=28			pre-heterologous boost T=104			post-heterologous boost T=108		
monkey	*98D209*	*106Q*	*113Q*	*98D209*	*106Q*	*113Q*	*98D209*	*106Q*	*113Q*	*98D209*	*106Q*	*113Q*	*98D209*	*106Q*	*113Q*	*98D209*	*106Q*	*113Q*
poolF	3110	263	404	1340	300	723	678	61	583	321	123	1438	204	192	326	1581	1525	1714
poolG	2115	642	1008	1070	316	2205	685	71	701	251	178	1758	166	106	625	1118	524	4238
poolH	373	72	19	358	43	43	424	24	42	51	23	18	92	45	55	413	58	211
pooll	103	37	347	80	36	531	237	39	169	12	35	485	66	79	376	459	85	2738
poolL	149	22	10	93	36	29	279	46	48	11	49	51	89	109	73	199	76	431
poolM	314	428	19	153	243	20	333	81	38	38	134	11	41	81	9	228	1440	227
DMSO	0	1	3	16	16	5	128	8	9	8	10	16	20	51	12	18	13	5

The efficiency of heterologous boost with chimp Ad vectors was evaluated in a second experiment. Cohorts of three monkeys were immunized at week 0 and week 4 with MRKAd5gag (10^10 vp/animal), MRKAd6NSmut (10^10 vp/animal) or with the combination of both vectors (10^10 vp/animal each vector) then boosted with the same immunogen at week 24 (homologous boost). Homologous boost was performed with the same immunogens; heterologous boost was performed with CV33gag, CV32 NSmut or with the two vectors in combination. The results provided in Table 9 summarize the number of spot-forming cells per million PBMC following incubation in absence (mock) or presence of HCV NS peptide pool.

The same cohorts were boosted again at week 51 with CV33gag (10^10 vp/animal), CV32NSmut (10^10 vp/animal) and with the combination of the two vectors (10^10 vp/animal each vector). The results provided in Table 9 further indicate that the homologous boost was not efficient since the responses are below the peak observed at week 4 after the injection of the first dose of vaccine. A strong boosting effect was measured by IFN-γ ELISPOT at week 54 after immunization with heterologous chimp vectors.

Table 9. Immunization with Chimp Ad vectors efficiently boost Gag and HCV NS-specific T cell response in monkeys immunized with MRK Ad5gag or MRK Ad6NSoptmut at 10^10 vp/animal

Vaccine	MRKAd5gag												CV33gag					
time point	post-dose 1 (T=4)			post-dose 2 (T=8)			pre-homol. boost (T=24)			post-homol. boost (T=28)			pre-heterol. boost (T=51)			post-heterol. boost (T=54)		
animal ID	00D105	00D076	00D299	00D105	00D076	00D299	00D105	00D076	00D299	00D105	00D076	00D299	00D105	00D076	00D299	00D105	00D076	00D299
poolF	18	35	60	16	29	14	37	76	40	37	8	14	37	27	44	43	44	70
poolG	16	23	49	4	28	31	54	95	106	81	2	46	36	27	37	84	**108**	109
poolH	45	51	57	18	31	42	55	88	55	47	11	32	69	36	60	85	58	120
pooll	21	21	48	4	26	11	19	54	26	38	6	6	22	11	32	33	26	24
poolL	15	21	58	9	31	20	71	183	128	**106**	6	27	61	21	65	28	45	44
poolM	39	24	49	26	14	49	38	93	39	59	6	19	62	23	38	27	19	14
Gag	**1764**	**2208**	**2762**	**574**	**1906**	**1959**	**391**	**935**	**702**	**2123**	**336**	**736**	**485**	**833**	**1384**	**4003**	**4333**	**3863**
DMSO	9	13	37	7	14	13	16	76	33	26	3	11	28	19	39	23	16	53

Vaccine	MRK Ad5gag + MRKAd6NSmut												CV33gag + CV32NSmut					
time point	post-dose 1 (T=4)			post-dose 2 (T=8)			pre-homol. boost (T=24)			post-homol. boost (T=28)			pre-heterol. boost (T=51)			post-heterol. boost (T=54)		
animal ID	00D088	00D099	00D240	00D088	00D099	00D240	00D088	00D099	00D240	00D088	00D099	00D240	00D088	00D099	00D240	00D088	00D099	00D240
poolF	**438**	**118**	**105**	**720**	**116**	**154**	**206**	**108**	**242**	**408**	**99**	**219**	**778**	**135**	**56**	**1701**	**1121**	**424**
poolG	21	**784**	**1483**	44	**362**	**940**	19	**234**	**548**	47	**781**	**844**	**78**	**363**	**265**	**228**	**3180**	**2770**
poolH	24	53	8	46	27	19	13	66	93	49	41	**87**	**115**	50	28	**97**	**291**	**104**
pooll	**83**	28	9	**90**	24	8	**16**	40	68	33	16	42	**56**	19	8	**165**	**145**	22
poolL	13	14	13	16	17	9	28	101	140	39	27	78	**59**	28	15	**137**	**815**	**463**
poolM	39	31	6	**101**	27	16	**21**	73	107	44	26	78	**114**	28	10	**219**	**109**	21
Gag	**2138**	**1044**	**1063**	**2260**	**505**	**819**	**454**	**241**	**456**	**1100**	**368**	**716**	**1542**	**237**	**161**	**4460**	**2908**	**1764**
DMSO	5	6	3	8	5	1	10	18	43	9	13	28	14	18	12	9	21	6

Vaccine	MRKAd6 NSmut												CV32NSmut					
time point	post-dose 1 (T=4)			post-dose 2 (T=8)			pre-homol. boost (T=24)			post-homol. boost (T=28)			pre-heterol. boost (T=51)			post-heterol. boost (T=54)		
animal ID	00D065	00D116	00D159	00D065	00D116	00D159	00D065	00D116	00D159	00D065	00D116	00D159	00D065	00D116	00D159	00D065	00D116	00D159
poolF	**139**	44	**82**	**92**	**121**	**63**	**62**	**116**	54	44	42	23	57	**85**	53	**313**	**385**	**261**
poolG	**154**	**253**	**119**	**77**	**156**	**108**	**93**	**165**	**126**	**104**	**59**	39	44	**198**	48	**196**	**764**	**559**
poolH	**1284**	41	**211**	**768**	35	**124**	**394**	84	**77**	24	**817**	48	**624**	31	**116**	**3758**	**90**	**925**
pooll	**302**	22	**1174**	**221**	16	**1069**	**134**	31	**561**	18	**133**	**478**	**84**	16	**362**	**485**	51	**2951**
poolL	28	16	48	35	32	21	141	113	78	19	48	17	46	33	46	**379**	**339**	**541**
poolM	**1329**	**1007**	36	**579**	**392**	30	**314**	**293**	43	**558**	**398**	22	**159**	**369**	33	**1278**	**1750**	16
Gag	15	9	7	13	5	2	36	33	36	9	23	14	16	8	10	37	9	26
DMSO	16	4	5	9	6	4	23	17	8	1	9	3	23	8	6	26	9	10

Example 12: Vaccination with a ChAd Vector Comprising a TAA Breaks Tolerance and Elicits a TAA-Specific T Cell Response in Monkeys

Experiments designed to determine whether chimpanzee adenoviral vectors are sufficiently immunogenic to break the tolerance to a self-antigen and to document the utility of chimpanzee vectors for boosting an immune response primed with a human adenoviral vector were performed in cohorts of four monkeys. Animals were immunized with three injection at week 0, 2 and 4 of Ad5DE1 RhCEA (10^11 vp), comprising the tumor associated antigen CEA, followed by vaccination at week 16, 18 and 20 with CV33DE1 RhCEA (10^11 vp). T cell response was measured by IFNγ ELISPOT with rhesus CEA peptides.

The results reported in figure 34, which provide the number of spot-forming cells per million PBMC following incubation in absence (DMSO) or in presence of rhesus CEA C and D peptides pools, establish that an immunization protocol based on vaccination with two different Ad serotypes leads to a sustained T cell response against CEA in non-human primates.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.